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STUDIES OF THE CHEMICAL NATURE OF THE  
 $\alpha$ -ADRENERGIC RECEPTOR

by

MAN SEN YONG



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SUBMITTED TO THE FACULTY OF GRADUATE STUDIES  
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UNIVERSITY OF ALBERTA  
FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Studies of the Chemical Nature of the  $\alpha$ -Adrenergic Receptor," submitted by Man Sen Yong in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Two labeling procedures were investigated for labeling the  $\alpha$ -adrenergic receptors of rabbit thoracic aorta with  $^{14}\text{C}$ -dibenamine hydrochloride. In procedure I the  $\alpha$ -adrenergic receptors were protected with epinephrine, while other receptors were masked with unlabeled dibenamine hydrochloride; the  $\alpha$ -adrenergic receptors were then labeled with  $^{14}\text{C}$ -dibenamine hydrochloride. In procedure II the  $\alpha$ -adrenergic receptors were protected with epinephrine while other receptors were labeled with  $^{14}\text{C}$ -dibenamine hydrochloride. Strips treated as described above have been designated experimental strips. As controls for both procedures, the experiments were repeated with the omission of epinephrine. With procedure II increments in radioactivity in the lipid-free residues and lipid extracts of the control strips as compared to the experimental strips were found. However, with procedure I the increment in radioactivity was confined to the lipid-free residue. Investigation of the lipid extracts by thin-layer chromatography did not support the claim that cephalin is the tissue receptor for epinephrine. Since  $\alpha$ -adrenergic blockade could be achieved in experiments in which  $^{14}\text{C}$ -dibenamine hydrochloride was located exclusively in the lipid-free residues a lipid is probably not involved in the  $\alpha$ -adrenergic receptor sites.

Strips exposed to  $^{14}\text{C}$ -dibenamine hydrochloride should be washed for 3 hr in order to remove the loosely bound radioactivity. The remaining firmly bound  $^{14}\text{C}$ -dibenamine hydrochloride is not exchangeable with unlabeled dibenamine hydrochloride. Labeling procedures I and II have been repeated with the modification that strips were washed for 3 hr. Procedure





II was found to be the preferred labeling procedure, and it was shown that phentolamine could be used in place of epinephrine in this procedure. After exposing strips to  $^{14}\text{C}$ -2-dibenzylaminoethanol hydrochloride all the radioactivity could be removed by washing for 6 hr. This observation showed that the retention of radioactivity by aortic strips exposed to  $^{14}\text{C}$ -dibenamine hydrochloride is not due to binding of its hydrolysis product, viz.,  $^{14}\text{C}$ -2-dibenzylaminoethanol.  $^{14}\text{C}$ -Dibenamine hydrochloride was found to be distributed between all subcellular components of rabbit aorta and we could therefore not decide in which subcellular component the  $\alpha$ -adrenergic receptors were located. Since epinephrine protects uptake sites in sympathetic nerve terminals in addition to  $\alpha$ -adrenergic receptors from combination with  $^{14}\text{C}$ -dibenamine hydrochloride a procedure was developed for denervation of rabbit aorta. With epinephrine as protecting agent in these modified aortic strips the significant increment in radioactivity associated with control strips as compared to experimental strips was confined to the lipid-free residue. This observation provided further evidence that the  $\alpha$ -adrenergic receptor is not lipid in nature. We have calculated that rabbit aorta contains  $360 \times 10^{14}$  receptors per gram dry weight.



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We dance 'round in a ring  
and suppose,  
But the Secret sits in the middle  
and knows.

— Robert Frost



We dance 'round the cell  
and suppose,  
But the Receptor sits inside  
and knows.

— With apologies to Robert Frost

"In our quest for the...receptor it is  
useful to consider the pithy couplet  
of Robert Frost...."

From Neil C. Moran (1967)



## CHAPTER I    GENERAL INTRODUCTION





### A. The Concept of Receptors

Ehrlich & Morgenroth (1910) made the interesting observation that certain substances containing sulfhydryl groups could effectively protect trypanosomes against a lethal dose of an organic arsenic derivative. This observation indicated that the toxicity of organic arsenicals was the result of interaction with essential sulfhydryl groups in trypanosomes. It was therefore suggested that drugs combine with a chemically defined area of the cell which was designated the drug receptor.

Langley (1905), who studied the effect of nicotine and curare on muscle and nerve endings, introduced the concept of "receptive substances" into pharmacology. He pictured the "receptive substances" as constituents of cells especially liable to change and "capable of setting the cell in function." He suggested that substances like nicotine, curare, atropine, etc., as well as the active constituents of internal secretions produced their effects by combining with "receptive substances."

Clark (1926) proposed a theory of drug receptor interaction based on the Langmuir adsorption isotherm. In this theory the following assumptions were made: (1) drug molecules have equal accessibility to all receptor sites; (2) drug molecules combine with receptors at a rate which is proportional to the concentration of drug in solution and to the number of free receptors in the tissue; (3) the intensity of response of a tissue is directly related to the number of receptors occupied by an active drug. When the response of the tissue reaches a maximum all the receptors are occupied by drug molecules. This theory explains many of the features of drug action on a cellular level but it offers no explanation why some drugs are stimulants (agonists), and others are antagonists. Moreover it



cannot account for the action of compounds, termed partial agonists, which although capable of stimulating tissues cannot produce as large a maximum response as that produced by agonists. In order to explain the above facts Stephenson (1956) suggested that different drugs may have different abilities to induce a response and consequently occupy different proportions of the receptors of a tissue when producing equal responses. This ability to induce a response is referred to as the "efficacy" of a drug. Thus in order for a drug to exert an effect it should have the ability to combine with a receptor (affinity), and the ability to induce a response subsequent to receptor occupation (efficacy). A similar modification of Clark's theory has been formulated by Ariëns (1954; 1964).

Paton (1961) proposed that excitation by a stimulant drug is proportional to the rate of drug-receptor combination rather than to the number of receptors occupied by the drug. According to Paton the value of the equilibrium constant ( $K_e$ ) determines the potency of a drug, whereas the value of the dissociation rate constant ( $K_d$ ) determines whether the drug is an agonist (high  $K_d$ ), a partial agonist (moderate  $K_d$ ), or an antagonist (low  $K_d$ ).

Evidence for the existence of drug receptors has been obtained by comparing the effects of optical isomers of drug molecules. Thus, where marked differences are observed in the pharmacological activity of the d- and l- isomers of a drug it is likely that the differences are due to the fit of the molecules to a complementary structure in the target cell, that is, a receptor site. One of the best examples of differences in activity observed with optical isomers is the study of Easson & Stedman (1933) with epinephrine. These authors showed that l-epinephrine had considerably

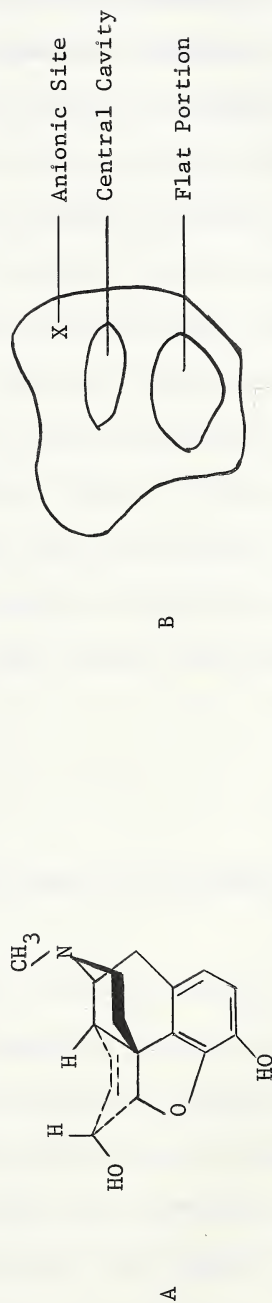
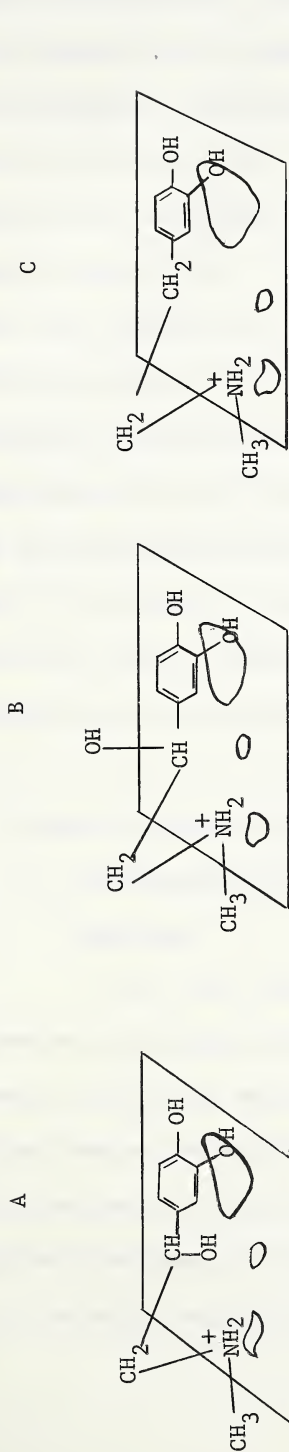


greater pharmacological activity than the d-isomer. This observation led them to suggest that the pharmacological activity of l-epinephrine depends upon the attachment of the amino, phenyl and  $\beta$ -hydroxyl groups to different portions of a specific receptor site (Fig. 1A). The lower activity of d-epinephrine is explained by the fact that only two point attachment to the receptor site is possible and the  $\beta$ -hydroxyl group is not involved in the binding (Fig. 1B). This idea is supported by the finding that deoxy-epinephrine (epinine) which has no  $\beta$ -hydroxyl group (Fig. 1C) has similar pharmacological activity to d-epinephrine.

Beckett & Casy (1954) studied a series of optical enantiomorphs of narcotic analgesics and found that the more active isomer of each enantiomorph pair had a spatial configuration related to that of D-(-)-alanine. On this basis and the results of studies on structure-activity relationships, they postulated that the analgesic receptor (Fig. 2B) consists of three essential sites: a flat portion for the binding of the aromatic ring; an anionic site for the nitrogen atom which acquires a proton at physiological pH; and a central cavity to accommodate a portion of the nitrogen-containing ring of morphine (Fig. 2A).

The studies of Furchgott (1954) provide the best evidence for the existence of receptors. He found that dibenamine hydrochloride produced irreversible blockade of the contractions elicited by adrenergic drugs, 5-hydroxytryptamine, histamine, and acetylcholine in rabbit aortic strips. Furthermore he reported the following observations: (1) when rabbit aortic strips were exposed to a very high concentration of an agonist and dibenamine hydrochloride was then added, the agonist was able to protect its receptors partially from the blockade produced by dibenamine











hydrochloride; however, the sites in the strip receptive to other agonists were not protected; (2) a reversible competitive antagonist towards a given agonist could protect against the dibenamine blockade of the response to the agonist. From these observations, Furchgott concluded that there are four distinct sets of contraction receptors in rabbit aorta, which are specific for adrenergic drugs, 5-hydroxytryptamine, histamine, and acetylcholine respectively. These studies of Furchgott have been extended to show that receptors for the above mentioned agonists exist in a variety of different organs (Innes, 1962a, 1962b). However, despite the usefulness of Furchgott's procedure, care is required in the interpretation of protection experiments for the following reasons (Waud, 1962): (1) the agonist used may have some affinity for receptors other than the specific type through which it initiates a response; (2) the agonist in sufficient concentration may still elicit a marked response even when a major fraction of its receptors are irreversibly blocked.

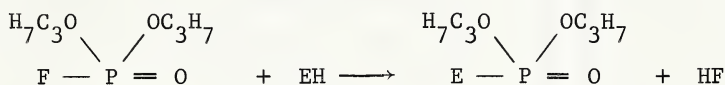
## B. Chemical Nature of Receptors

### i. Relationships Between the Active Center of an Enzyme and Drug Receptors

Many drugs whose pharmacological action is specific and structure-dependent are thought to display a high degree of molecular complementarity towards the site at which they act. It is therefore possible that receptors are macromolecules capable of interacting with a drug in a manner analogous to the enzyme-substrate interaction. In fact the information obtained from studies of enzymes and other macromolecules has been applied to interpret the interaction between drug and receptor.



Organophosphorus compounds have been utilized to study the active centers of esterases and proteases. The organophosphorus inhibitor, diisopropylfluorophosphate (DFP), reacts stoichiometrically with  $\alpha$ -chymotrypsin (EH) (Balls & Jansen, 1952) as depicted below.



Schaffer, May & Summerson (1954) labeled the active center of eel cholinesterase with  $^{32}\text{P}$ -labeled DFP and isolated serine phosphoric acid from the acid hydrolysate of the phosphorylated enzyme. Studies of other phosphorylated esterases showed that radioactive phosphorus is always bound to one of the serine residues in the polypeptide chain. Cohen & Warringa (1953) used the following method to specifically label the active center of purified ox red cell cholinesterase: the active center of the enzyme was first protected with the reversible competitive inhibitor, butyrylcholine, and non-specific sites were then phosphorylated with unlabeled DFP. After exhaustive dialysis, the enzyme which was still active was treated with  $^{32}\text{P}$ -labeled DFP (Fig. 3). This procedure has been adapted for the labeling of drug receptors and will be described in detail later in this thesis.

## ii. Attempts to Identify and Isolate Drug Receptors

While a great deal of work has been carried out on the chemical nature of the active sites of enzymes, few studies have been reported on the chemical nature of drug receptors. For this reason the reality of receptors has been questioned by many investigators. Since the interpretation of the action of many drugs, especially those concerned with neural transmission, is



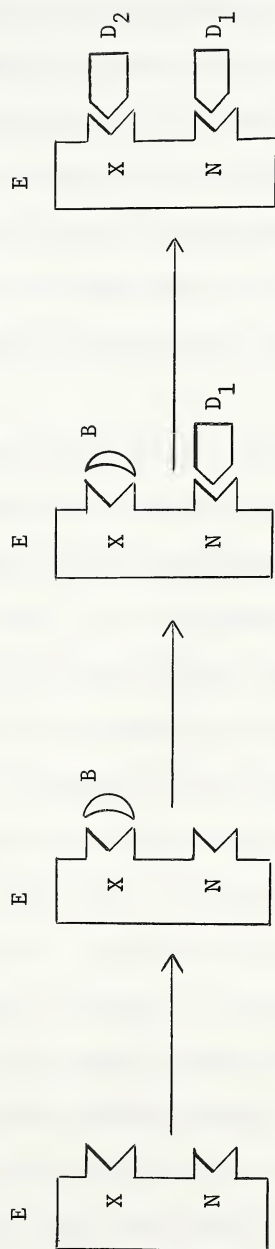


Fig. 3. Labeling of the Active Center of Ox Red Cell Cholinesterase by  $^{32}\text{P}$ -labeled DFP. X, Active center of enzyme; N, non-specific site of enzyme; B, butyrylcholine molecule;  $\text{D}_1$ , unlabeled DFP molecule;  $\text{D}_2$ ,  $^{32}\text{P}$ -labeled DFP molecule.



based on the idea that specific receptors exist, the progress of modern pharmacology is dependent upon the demonstration of the existence of receptors and the elucidation of their chemical nature.

Waser & Lüthi (1956) injected mice with a minimal lethal dose of radioactive curarine and by means of radioautography showed that this agent was located over the endplate regions of the diaphragm. A similar result was obtained with radioactive decamethonium. The results provided evidence that neuromuscular blocking agents are fixed in the endplate through interaction with specific cholinergic receptors (Waser & Lüthi, 1957; Waser, 1961).

The ability of the neuromuscular blocking agent, gallamine, to form a non-dialyzable complex with a protein component of an aqueous extract of the electric organ of *Electrophorus electricus* L. was first observed by Chagas et al. (1958). In subsequent studies a specific component of the aqueous extract of the electric organ was shown to possess very strong binding capacity for gallamine and other quaternary ammonium compounds. This component was shown to be an acid mucopolysaccharide, and the possibility that it might represent receptor material has been considered (Hassón & Chagas, 1959; 1961). Ehrenpreis (1959) treated the aqueous extract of the electric organ of an electric eel with curare and by this means isolated protein material. A correspondence was found between the binding of quaternary ammonium compounds to this protein and their activity as neuromuscular blocking agents. It was therefore suggested that this protein is the acetylcholine receptor (Ehrenpreis, 1961). It must be stressed, however, that the capacity of a macromolecule to bind a drug does not necessarily indicate that the macromolecule is the drug





receptor. Moreover, a further limitation in the use of curare to elucidate the chemical nature of the acetylcholine receptor is the fact that a complex formed between curare and the receptor will be labile. Consequently it is likely that a drug-receptor complex formed when the drug is added to an organ will break down during the course of the isolation process. It will be recalled that the elucidation of the nature of the active center of enzymes required the use of an inhibitor which could form a covalent bond. It is therefore likely that a successful approach to the study of drug receptors will require a drug which forms a covalent bond with the receptor.

Woolley & Gommi (1964; 1966) in the course of their studies on the 5-hydroxytryptamine (5-HT) receptor reported the following observations:

(1) the 5-HT receptor in the rat uterus and stomach could be destroyed by exposing these organs to a small amount of a mixture of neuraminidase and ethylenediaminetetraacetic acid (EDTA); (2) either crude lipids extracted from rat stomach or gangliosides obtained from bovine brain could partially restore the sensitivity of neuraminidase-EDTA treated organs to 5-HT. These observations led Woolley to suggest that gangliosides might be a component of the 5-HT receptor. Offermeier & Ariëns (1966) have reported that they could not confirm these results.

### C. The Adrenergic Receptors

#### i. Classification of Adrenergic Receptors

Dale (1906) pointed out that the ergot alkaloids prevented the excitatory activity of epinephrine but had no effect on its inhibitory activity. This led to the suggestion that there is one class of adeno-tropic receptors subserving excitation of effector cells and a second class



subservient inhibition. However, experiments on anesthetized animals and on isolated tissues indicated that adrenotropic receptors could not be classified simply as excitatory or inhibitory (Ahlquist, 1948). The evidence for this conclusion was based on the following experiment: the responsiveness of a variety of tissue preparations to a series of six structurally related sympathomimetic amines was determined. The comparative potencies of the amines suggested that there were two distinct types of adrenotropic receptors, one of which was designated the  $\alpha$ - and the second the  $\beta$ - receptor. The  $\alpha$ -adrenotropic receptor was associated with most of the excitatory functions and one important inhibitory function, viz., intestinal relaxation. The  $\beta$ -adrenotropic receptor was associated with most of the inhibitory functions and one excitatory function, viz., myocardial stimulation. Only the responses mediated through the  $\alpha$ -receptor could be blocked by the then known adrenergic blocking agents. For this reason these blocking agents were referred to as  $\alpha$ -adrenergic receptor blocking agents.

#### ii. 2-Halogenoethylamines

A series of 2-halogenoethylamines of which N-(2-chloroethyl)-dibenzylamine hydrochloride (dibenamine hydrochloride; Fig. 4) may be considered the prototype, possess  $\alpha$ -adrenergic blocking activity. Nickerson & Goodman (1947) injected dibenamine hydrochloride into anesthetized cats and made the following observations: (1) the pressor response elicited by epinephrine or by splanchnic nerve stimulation was reversed; (2) the effects of epinephrine on the myocardium were not affected; (3) the blockade which was of prolonged duration could be antagonized by prior



administration of sodium thiosulfate. Harvey & Nickerson (1953) attributed the pharmacological activity of dibenamine and its congeners to the formation at physiological pH of a highly reactive and unstable ethylenimmonium (EI) ion. This idea was based on the known formation of EI ions by the structurally related nitrogen mustards. The following evidence supports the hypothesis that the EI ion is the molecular species responsible for  $\alpha$ -adrenergic blockade: (1) antagonism of the action of 2-halogenoethylamines by sodium thiosulfate may be explained by the known reaction between sodium thiosulfate and EI ions (Graham, 1962); (2) 2-halogenoethylamines which are unable to form EI ions are inactive as adrenergic blocking agents (Graham & Lewis, 1953); (3) isolated EI derivatives of several dibenamine analogues may be even more potent than the parent compounds in producing  $\alpha$ -adrenergic blockade (Graham, 1957; 1960).

Since it is well known that EI ions are highly reactive substances and react readily with nucleophilic groups, Nickerson (1957, 1962) interpreted the prolonged blockade produced by dibenamine and congeners to the formation of a covalent bond between the  $\alpha$ -adrenergic receptor and the EI ion as shown in Fig. 4. However, the following workers have questioned the above interpretation of the action of dibenamine and congeners: (1) Axelrod, Aronow & Brodie (1952) showed that dibenamine and congeners are stored in fat depots and attributed the prolonged action of these compounds to slow release from these depots; (2) Moran et al. (1967) studied the distribution of the  $\alpha$ -adrenergic blocking agent,  $^3\text{H}$ -labeled N-(2-bromoethyl)-N-ethyl-1-naphthylmethylamine ( $^3\text{H}$ -SY.28) (Fig. 5), and its corresponding alcohol in rats. The prolonged retention of radioactivity following  $^3\text{H}$ -SY.28 administration was attributed to the high binding capacity of tissues for the alcohol derived



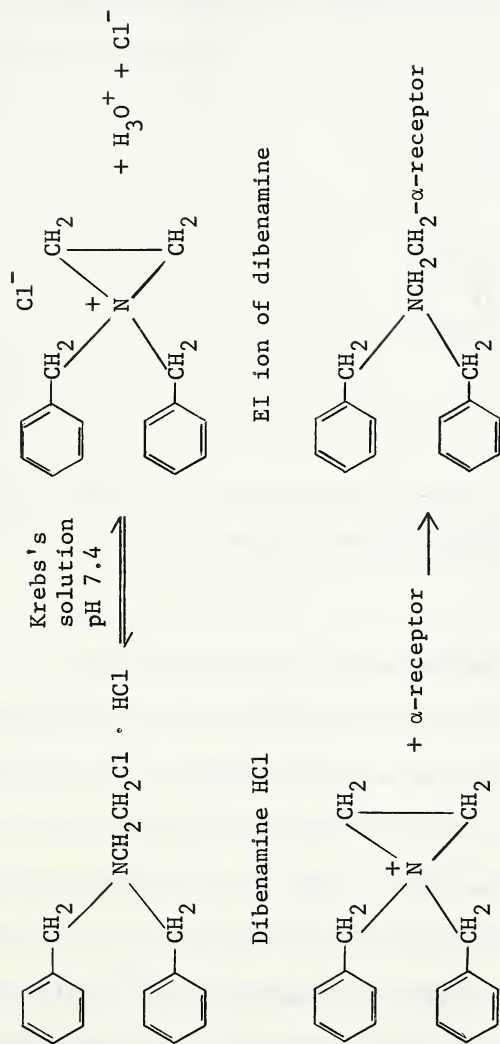


Fig. 4. Formation of Ethyleniminium (EI) ion from dibenamine and its reaction with the  $\alpha$ -adrenergic receptor.





by hydrolysis of  $^3\text{H}$ -SY.28 at physiological pH rather than to covalently bound  $^3\text{H}$ -SY.28; (3) Olivares, Smith & Aronow (1967) showed that propranolol, a  $\beta$ -adrenergic blocking agent, could partially reverse the blockade produced by phenoxybenzamine in rabbit aortic strips. For this reason they suggested that phenoxybenzamine and congeners are not covalently linked to the  $\alpha$ -adrenergic receptors.

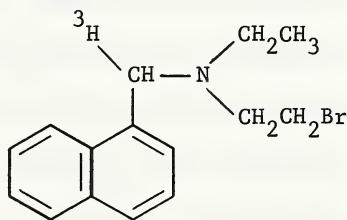


Fig. 5. The Chemical Structure of  $^3\text{H}$ -Labeled N-(2-bromoethyl)-N-ethyl-1-naphthylmethanamine ( $^3\text{H}$ -SY.28)

This controversy on the mechanism of action of dibenamine and congeners led to considerable study of the problem. The experiments described below provide strong support for the original interpretation of the action of these compounds (Nickerson, 1957; 1962): (1) cross-circulation experiments in dogs (Hunt, 1949) and in cats (Agarwal & Harvey, 1956) showed that slow release of phenoxybenzamine from adipose tissue plays a minor role in the maintenance of adrenergic blockade; (2)  $\alpha$ -adrenergic blockade induced by dibenamine hydrochloride and congeners in isolated smooth muscle preparations is maintained for a prolonged period despite frequent washings (Furchgott, 1954; Nickerson, 1962). Clearly the prolonged



blockade of excitatory responses to norepinephrine induced by dibenamine and congeners could not be explained on the basis of slow release of the blocking agent from fatty depots.

### iii. Proposals on the Nature of $\alpha$ -Adrenergic Receptors

Belleau (1958) suggested that there are two major binding sites on  $\alpha$ -adrenergic receptors. One of these binding sites (site A; Fig. 6A) is concerned with binding the amine function of epinephrine in its protonated form and the second site (site B) contributes to the efficacious binding of the drug. He further suggested that interaction of epinephrine with site A triggers an excitatory response. Evidence was presented that site A was probably a carboxylate or phosphate anion. The minor binding sites (P and M) were thought to be involved with binding of phenolic hydroxyl groups. The EI ion of dibenamine is depicted as combining with this receptor as shown in Fig. 6B. Belleau pointed out that the carbon atoms of the three-membered ring of the EI ion have carbonium ion character and hence affinity for the negatively charged site A. Alkylation of site A is believed to take place with production of irreversible blockade (Fig. 6C).

Bloom & Goldman (1966) have visualized the  $\alpha$ -adrenergic receptor as a complex between magnesium-activated adenosinetriphosphatase (ATPase) and its substrate adenosinetriphosphate (ATP). An agonist acting on this receptor is thought to facilitate the utilization of the substrate, ATP, while antagonists of the 2-halogenoalkylamine type are believed to inhibit utilization of the substrate.

Robison, Butcher & Sutherland (1967) have proposed the following



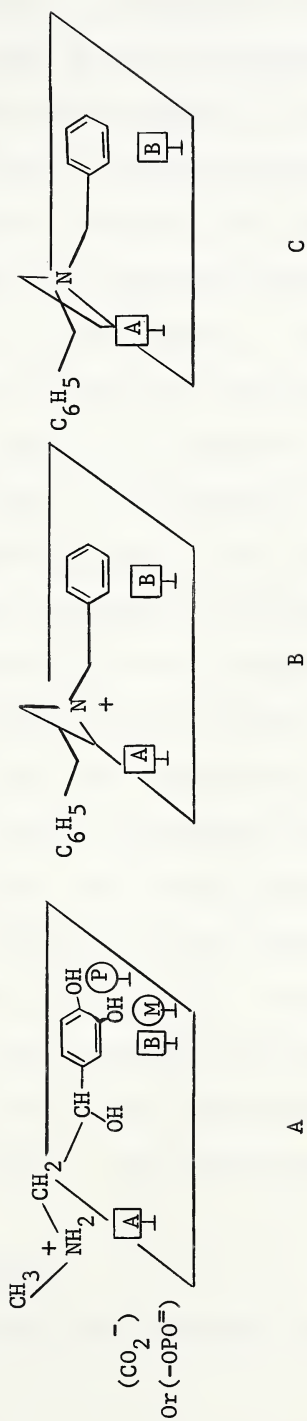


Fig. 6. Postulated  $\alpha$ -Adrenergic Receptor (Belleau, 1958). A. Interaction of epinephrine with the receptor. B. Interaction of EI ion of dibenamine with the receptor. C. Alkylation of site A of the receptor.



model of the adenylyl cyclase molecule (Fig. 7A) which catalyzed the conversion of ATP to adenosine 3',5'-phosphate (cyclic AMP). The molecule which is considered to exist in the cell membrane is depicted as being composed of at least two distinct subunits, a regulatory subunit, facing the extracellular fluid, and a catalytic subunit, the active center of which is in contact with the interior of the cell. It is suggested that epinephrine interacts with the regulatory subunit which in turn influences the configuration of the catalytic subunit. These workers have suggested that enough circumstantial evidence is available to warrant consideration of the hypothesis that both  $\alpha$ - and  $\beta$ -receptors may be related to the regulatory subunit of the enzyme. One possibility considered by Robison, Butcher & Sutherland (1967) is that  $\alpha$ - and  $\beta$ -receptors are two different regulatory subunits which are bound to the catalytic subunit of adenylyl cyclase (Fig. 7B). Interaction of the  $\alpha$ -receptor with epinephrine is suggested to lead to a decrease in adenylyl cyclase activity and contraction of smooth muscle. On the other hand interaction of the  $\beta$ -receptor with epinephrine is thought to lead to stimulation of adenylyl cyclase activity and relaxation of smooth muscle. The second possibility is that the  $\alpha$ - and  $\beta$ -receptors are bound to separate catalytic subunits, which may be located in different parts of the cell (Fig. 7C). According to these workers "effective interaction with epinephrine leads in either case to a stimulation of adenylyl cyclase activity, and compartmentalization is invoked to explain those cases in which  $\alpha$ - and  $\beta$ -effects are opposite."

#### D. Statement of the Problem

Although there has been widespread speculation on the nature of





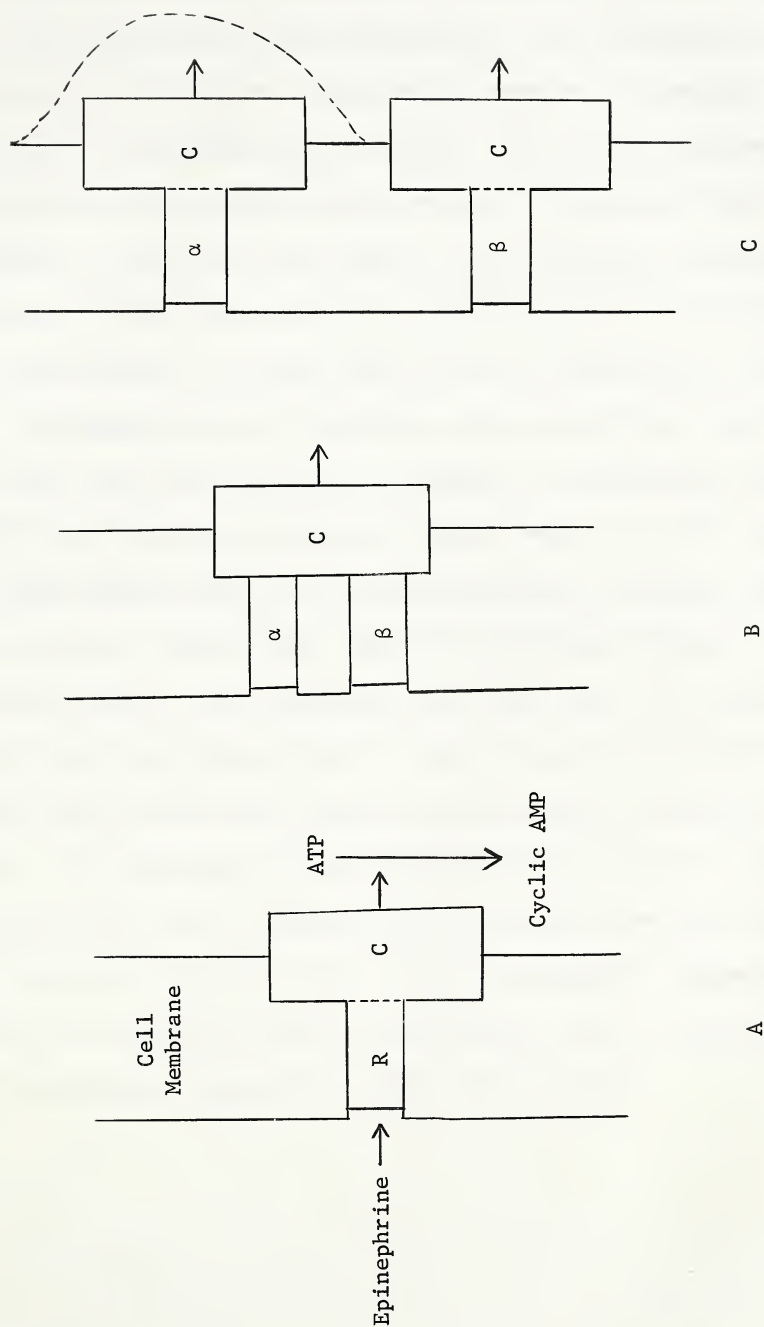


Fig. 7. Model of Adenyl Cyclase as Related to Adrenergic Receptors (Robison, Butcher & Sutherland, 1967). A, Model of adenyl cyclase; R, regulatory subunit; C, catalytic subunit. B. α- and β-receptor as two different regulatory subunits bound to one catalytic subunit. C. α- and β-receptors as two different regulatory subunits bound to separate catalytic subunits.



the  $\alpha$ -adrenergic receptors there is no evidence as to their chemical nature. The objectives of this study were: (1) to establish appropriate conditions for labeling the  $\alpha$ -adrenergic receptors of vascular smooth muscle with  $^{14}\text{C}$ -dibenamine hydrochloride; (2) to devise procedures for the isolation of the complex formed between  $^{14}\text{C}$ -dibenamine and the receptor. The approach to this study was based on the following considerations:

(1) Furchgott (1954) identified four distinct sets of contraction receptors in the smooth muscle of rabbit aorta, which are specific for adrenergic drugs, 5-hydroxytryptamine, histamine, and acetylcholine respectively. He showed that when this preparation is exposed to epinephrine bitartrate ( $1 \times 10^{-4}$ ) for 5 min and dibenamine hydrochloride ( $3 \times 10^{-6}$ ) added, the latter drug combines with the 5-hydroxytryptamine, histamine and acetylcholine receptor, respectively, while the adrenergic receptor is protected, at least partially, from combination with this drug; (2) Gaddum (1962) suggested that this method could be used to mask the 5-hydroxytryptamine, histamine and acetylcholine receptors with unlabeled dibenamine hydrochloride; the adrenergic receptor which would be protected under these conditions could then be labeled with radioactive dibenamine hydrochloride. It was proposed to carry out the series of experiments suggested by Gaddum and see if this approach could be successfully used for specific labeling of the  $\alpha$ -adrenergic receptors of rabbit aortic strips.



CHAPTER II LABELING OF THE  $\alpha$ -ADRENERGIC RECEPTORS OF RABBIT  
AORTA WITH  $^{14}\text{C}$ -DIBENAMINE HYDROCHLORIDE



A. Radiochemical Purity and Pharmacological Activity of  $^{14}\text{C}$ -Labeled  
N-(2-chloroethyl)-dibenzylamine (Dibenamine) Hydrochloride

Introduction

$^{14}\text{C}$ -Dibenamine hydrochloride, m.p. 188-190°, was prepared from benzyl-7- $^{14}\text{C}$ -chloride (0.25 mc/m-mole) in this laboratory (Parulekar, 1965) by a method previously used by Gump & Nikawitz (1950) to prepare the unlabeled compound (Fig. 8). When mixed with a sample of dibenamine hydrochloride (Smith, Kline & French; SKF) it had m.p. 188-190°. The infrared spectrum of the  $^{14}\text{C}$ -dibenamine hydrochloride was identical in all respects with that of dibenamine hydrochloride (SKF). It had a specific activity of 0.48 mc/m-mole.

It is well known that radiation can produce profound chemical changes and thus lead to the formation of radiochemical impurities (Tolber & Lemmon, 1955). For this reason the radiochemical purity of the  $^{14}\text{C}$ -dibenamine hydrochloride which had been prepared approximately one year prior to the beginning of this study was assessed by means of reverse isotope dilution analysis. To check the pharmacological properties of this preparation its capacity to block the effects of epinephrine on rabbit aortic strips was compared to that of unlabeled dibenamine hydrochloride (SKF).

Experimental

i. Reverse Isotope Dilution Analysis of  $^{14}\text{C}$ -Dibenamine Hydrochloride

$^{14}\text{C}$ -Dibenamine hydrochloride (1 mg; 0.48 mc/m-mole) was mixed with unlabeled dibenamine hydrochloride (500 mg) and dissolved in ethanol





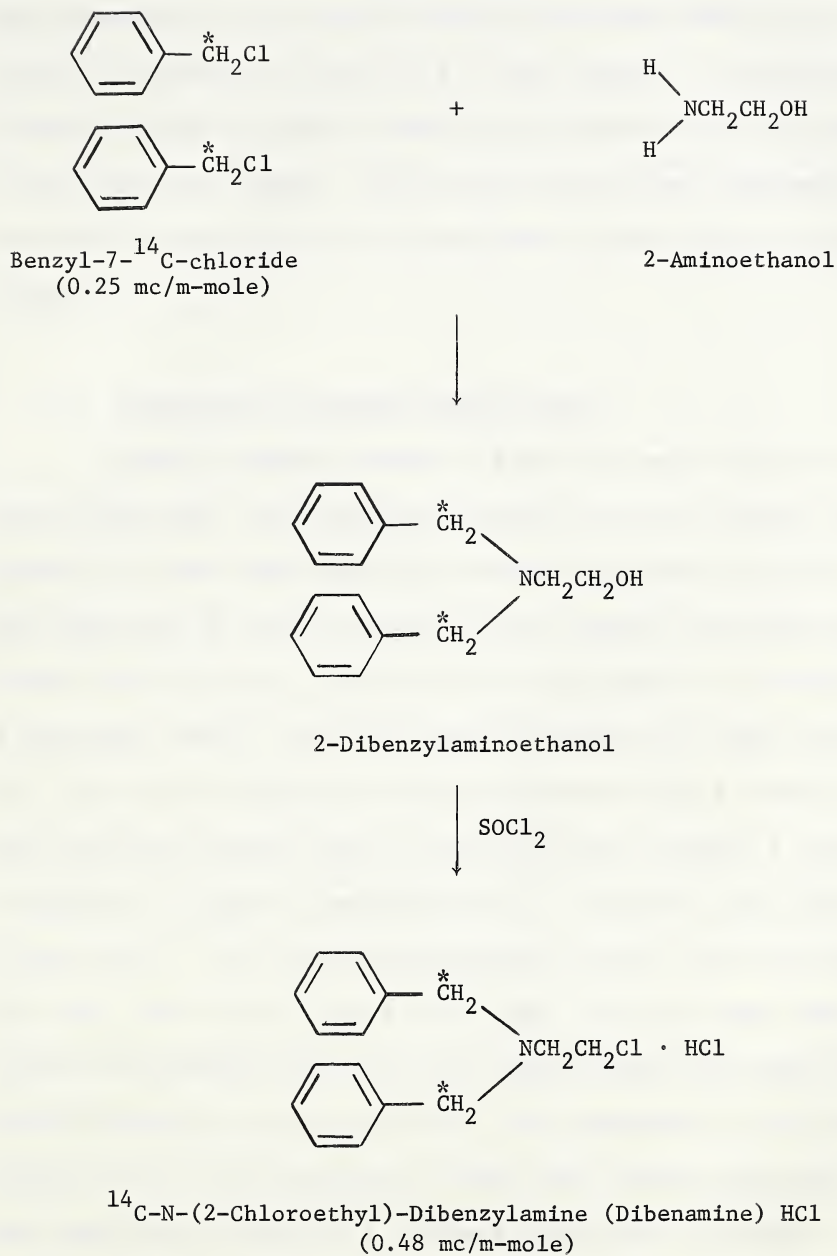


Fig. 8. Chemical Synthesis of  $^{14}\text{C}$ -N-(2-Chloroethyl)-Dibenzylamine (Dibenamine) Hydrochloride.



(10 ml). The radioactivity present in measured aliquots of this solution was determined in the Nuclear Chicago Unilux Model 6850 liquid scintillation counter as described in section B of this chapter. The solution of  $^{14}\text{C}$ -dibenamine hydrochloride in ethanol was evaporated and the residue recrystallized twice from ethanol. There was no significant difference in radioactivity between the twice recrystallized product and the original mixture (Table I).

#### ii. Preparation of Rabbit Aortic Strips

Rabbits weighing between 1.5 and 3 kg were killed by a blow on the back of the neck. The descending thoracic aorta was quickly removed and placed in a Petri dish containing Krebs's bicarbonate solution aerated with 95% oxygen and 5% carbon dioxide. After removing the adipose and connective tissue from the aorta, a helical strip was prepared as follows (Furchgott & Bhadrakom, 1953): The aorta was held between the thumb and fingers of the left hand and gradually rotated and moved forward towards a pair of sharp-pointed scissors held in the right hand to permit a continuous spiral incision at an angle of approximately  $15^\circ$  relative to the long axis of the intact aorta. A cut strip (approximately 30 mm x 5 mm) was mounted as a loop (Fig. 9A) or as a single strip (Fig. 9B) in an organ bath containing Krebs's bicarbonate solution (15 ml) aerated with 95% oxygen and 5% carbon dioxide mixture to give a pH of 7.4. The temperature of the bath was maintained at  $37.5^\circ$  by circulation of warm water through the outer jacket of the organ bath by means of a constant temperature circulator. The tension of the tissue was maintained at 2 g throughout the experiment.



TABLE I. ISOTOPE DILUTION ANALYSIS OF  
 $^{14}\text{C}$ -DIBENAMINE HYDROCHLORIDE

Sample	Specific Activity <sup>†</sup> of Diluted $^{14}\text{C}$ -Dibenamine HCl
Original Mixture*	6399 $\pm$ 59 (3)
After Two Recrystallizations	6496 $\pm$ 70** (3)

\* Original mixture was prepared by mixing 1 mg of  $^{14}\text{C}$ -dibenamine HCl (specific activity 0.48 mc/m-mole) with 500 mg of unlabeled dibenamine HCl.

\*\* Not significantly different from the original mixture at 0.05 level.

<sup>†</sup> Dis/min/mg.



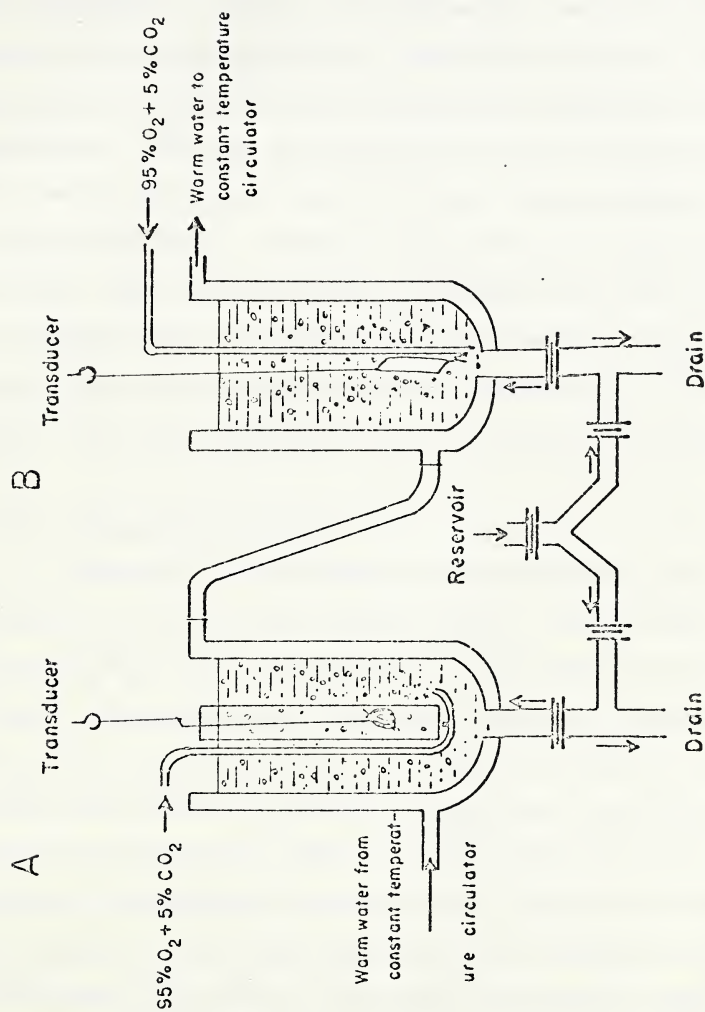


Fig. 9. Apparatus Used for Mounting Rabbit Aortic Strips.

A. Aortic strip mounted as a loop. B. Aortic strip mounted as a single strip.





### iii. Calibration of Recording Instrument

Aortic strips were mounted as single strips (Fig. 9B) and the contraction recorded by means of force and displacement transducers (Grass FT.03) connected to a Grass model 5P1 polygraph. The driver amplifier was first calibrated to yield a 2 cm deflection per 200 millivolt input from a companion plug-in preamplifier. The bridge circuit of the transducer was then balanced with the balance voltage and sensitivity control knobs in the preamplifier. The base line position was selected and the transducer loaded with a series of weights from 1 to 4 g. The sensitivity of the instrument was adjusted so that a weight of 1 g produced an upward deflection of 1 cm of the pen. The response of the instrument was linear in the 1 to 4 g range. The transducer was then loaded with a 2 g weight, the pen adjusted to the base line and the instrument again calibrated with a series of 1 to 4 g weights. The chart speed was set at 0.25 cm/sec throughout the experiment.

### iv. Drugs and Solutions

The Krebs's bicarbonate solution used in this study contained 0.116 M NaCl, 0.0046 M KCl, 0.0024 M  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.001 M  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.001 M  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 0.021 M  $\text{NaHCO}_3$ , and 0.045 M dextrose. The volume of Krebs's bicarbonate solution in the organ bath was 14.8 ml. Drugs were made up to a final volume of 0.2 ml before addition to the bath. The following drugs were used: l-epinephrine bitartrate (Nutritional Biochemicals); dibenamine hydrochloride (SKF); and  $^{14}\text{C}$ -dibenamine hydrochloride. A stock solution of l-epinephrine bitartrate (7.5 mg/ml) was freshly prepared in normal saline for each experiment and used to prepare a series of dilutions of this drug. Solutions of dibenamine hydrochloride and  $^{14}\text{C}$ -dibenamine hydrochloride were always freshly prepared 5 min before addition to the bath with normal saline containing 0.01 M HCl. All drug solutions were kept in an icebath for the duration of an experiment. Drug concentrations, unless otherwise specified, are expressed as grams of salt per milliliter bath fluid.



v.  $\alpha$ -Adrenergic Receptor Blocking Activity of  $^{14}\text{C}$ -Dibenamine Hydrochloride

In a series of experiments the responses of 16 rabbit aortic strips to several concentrations of epinephrine were recorded. From the average of these responses a cumulative dose-response curve was constructed.

Eight of the above rabbit aortic strips were then exposed to the following concentrations of  $^{14}\text{C}$ -dibenamine hydrochloride for 20 min: strips 1 and 2,  $3 \times 10^{-8}$ ; strips 3 and 4,  $1.2 \times 10^{-7}$ ; strips 5 and 6,  $3 \times 10^{-7}$ ; strips 7 and 8,  $3 \times 10^{-6}$ . The strips were then washed and the responses of these strips to several concentrations of epinephrine recorded. From the data the cumulative dose-response curves were plotted (Fig. 10B). The above experiment was repeated using the remaining eight rabbit aortic strips and substituting unlabeled dibenamine hydrochloride for  $^{14}\text{C}$ -dibenamine hydrochloride. The results are recorded in Fig. 10A.

It is apparent from these results that the degree of  $\alpha$ -adrenergic blockade produced in isolated rabbit aortic strips by  $^{14}\text{C}$ -dibenamine hydrochloride is similar to that produced by unlabeled dibenamine hydrochloride. Thus both labeled and unlabeled dibenamine hydrochloride at a concentration of  $3 \times 10^{-8}$  caused a slight shift of the dose-response curve to the right without an effect on the maximum response. At higher concentrations of labeled and unlabeled dibenamine hydrochloride the dose-response curve was shifted further towards the right and the maximum response attainable was decreased; complete blockade of the response of aortic strips to epinephrine was attained with labeled and unlabeled dibenamine hydrochloride at a concentration of  $3 \times 10^{-6}$ . This blockade was maintained throughout a period of 3 hr and strips became slightly responsive to a very high concentration of epinephrine ( $1 \times 10^{-4}$ ) after a period of 6 hr had elapsed.

It was therefore concluded that the  $^{14}\text{C}$ -dibenamine hydrochloride which has the same melting point, and infrared spectrum, as dibenamine hydrochloride (SKF) is indistinguishable as an  $\alpha$ -adrenergic receptor blocking



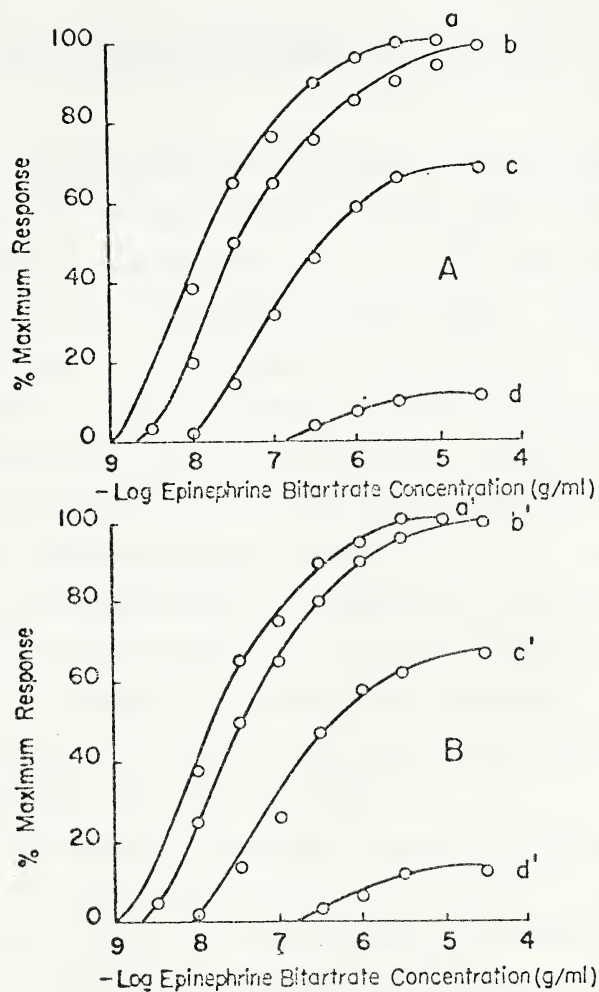


Fig. 10.  $\alpha$ -Adrenergic Receptor Blocking Activity of Unlabeled Dibenamine Hydrochloride (A) and  $^{14}\text{C}$ -Dibenamine Hydrochloride (B). Concentrations of these agents: a, a', 0; b, b',  $3 \times 10^{-8}$ ; c, c',  $1.2 \times 10^{-7}$ ; d, d',  $3 \times 10^{-7}$ .



agent from unlabeled dibenamine hydrochloride (SKF).

## B. Labeling of the $\alpha$ -Adrenergic Receptors (Procedure I)

### Introduction

Our first experiments were directed to repeating the receptor protection experiments of Furchgott (1954) on rabbit aortic strips. We then turned our attention to labeling the strips with  $^{14}\text{C}$ -dibenamine hydrochloride according to the procedure suggested by Gaddum (1962). Our experimental design was as follows: A rabbit aortic strip containing adrenergic, 5-hydroxytryptamine, histamine, and acetylcholine receptors as depicted in Fig. 11a was treated with epinephrine to protect the adrenergic receptor (Fig. 11b). Unlabeled dibenamine hydrochloride was then added to mask the 5-hydroxytryptamine, histamine, and acetylcholine receptors (Fig. 11c). After washing the tissue to remove the epinephrine (Fig. 11d) the strip was treated with  $^{14}\text{C}$ -dibenamine hydrochloride to label the  $\alpha$ -adrenergic receptor (Fig. 11e). A strip treated in this manner is designated the experimental strip. As a control the experiment was repeated, omitting the protecting dose of epinephrine in the first step (Fig. 11a',c',d',e').

After labeling the  $\alpha$ -adrenergic receptor by this method it was expected that experimental strips would contain significantly greater radioactivity than control strips. It was desirable to determine that this was in fact the case and our next experiments were directed to determining the total radioactivity in experimental and control strips. While this work was in progress, reports appeared of a similar study using dibenamine hydrochloride randomly labeled with tritium. In these reports (Dikstein, Silber & Sulman, 1963; Dikstein & Sulman, 1965) it was implied that cephalin is the tissue receptor for epinephrine. For this reason the amount of radioactivity in lipid extracts and lipid-free residues of aortic strips was determined. The sum of the radioactivity in the lipid extract and lipid-free residue of a strip was a measure of the total radioactivity of the strip.







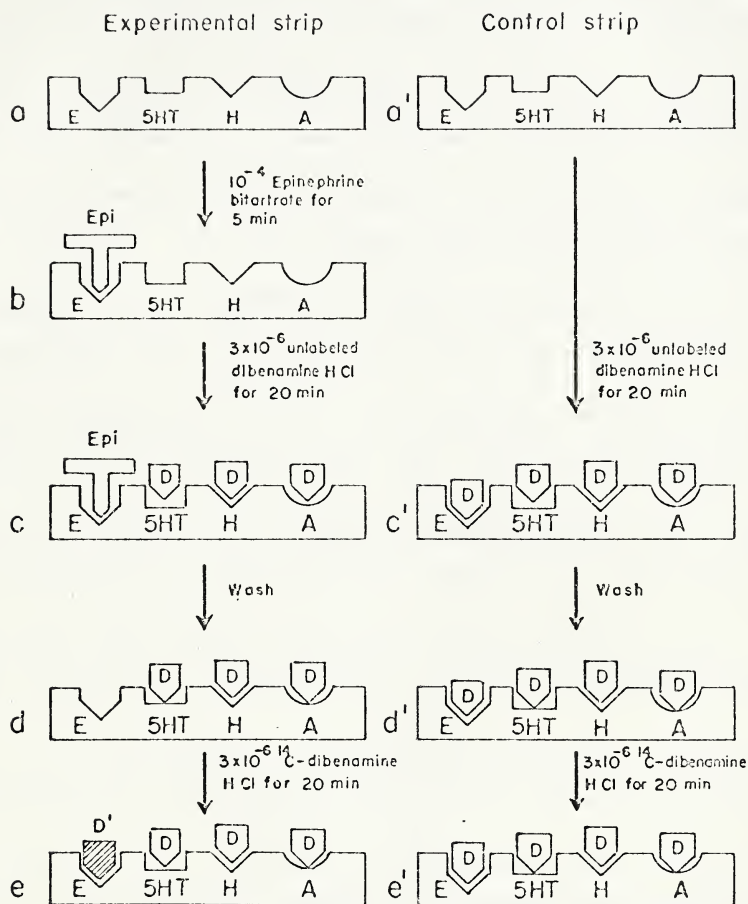


Fig. 11. Schematic Representation of Labeling Procedure I. E,  $\alpha$ -adrenergic receptor; 5HT, 5-hydroxytryptamine receptor; H, histamine receptor; A, acetylcholine receptor; D, unlabeled dibenamine hydrochloride; D',  $^{14}\text{C}$ -dibenamine hydrochloride; Epi, epinephrine.



## Experimental

### i. Labeling the $\alpha$ -Adrenergic Receptors of Rabbit Aortic Strips

Rabbit aortic strips were mounted as loops (Fig. 9A) as follows: The two ends of a single strip were hooked together and the hook attached by means of a thread to a Statham Model G7A transducer. All other conditions were kept identical to those reported in section A of this chapter. The responses of aortic strips to several concentrations of epinephrine, 5-hydroxytryptamine creatinine sulfate (Nutritional Biochemicals), histamine phosphate (Fisher Scientific), and acetylcholine bromide (Eastman Organic Chemicals) were recorded (Fig. 12A,B,C,D). The strips were then treated with epinephrine ( $1 \times 10^{-4}$ ) for 5 min and unlabeled dibenamine hydrochloride ( $3 \times 10^{-6}$ ) added (Fig. 12E). After a further 20 min period the strips were washed three times with epinephrine ( $1 \times 10^{-4}$ ) and three times with bath fluid. The strips were washed with bath fluid at 15 min intervals until they relaxed completely (approximately  $1\frac{1}{2}$  hr). The responses of strips to various concentrations of epinephrine and other drugs were then redetermined. As expected, the strips retained considerable responsiveness to epinephrine (Fig. 12F), slight responsiveness to acetylcholine, but were unresponsive to 5-hydroxytryptamine and histamine (Fig. 12G). The strips were exposed to  $^{14}\text{C}$ -dibenamine hydrochloride ( $3 \times 10^{-6}$ ) for 20 min and then washed six times with bath fluid. After this treatment the strips were unresponsive to epinephrine ( $1 \times 10^{-4}$ ), showing that the  $\alpha$ -adrenergic receptors had interacted with  $^{14}\text{C}$ -dibenamine hydrochloride. Strips treated as above have been designated experimental strips. As controls, the experiments were repeated with the omission of the protecting dose of epinephrine ( $1 \times 10^{-4}$ ) in the first step.



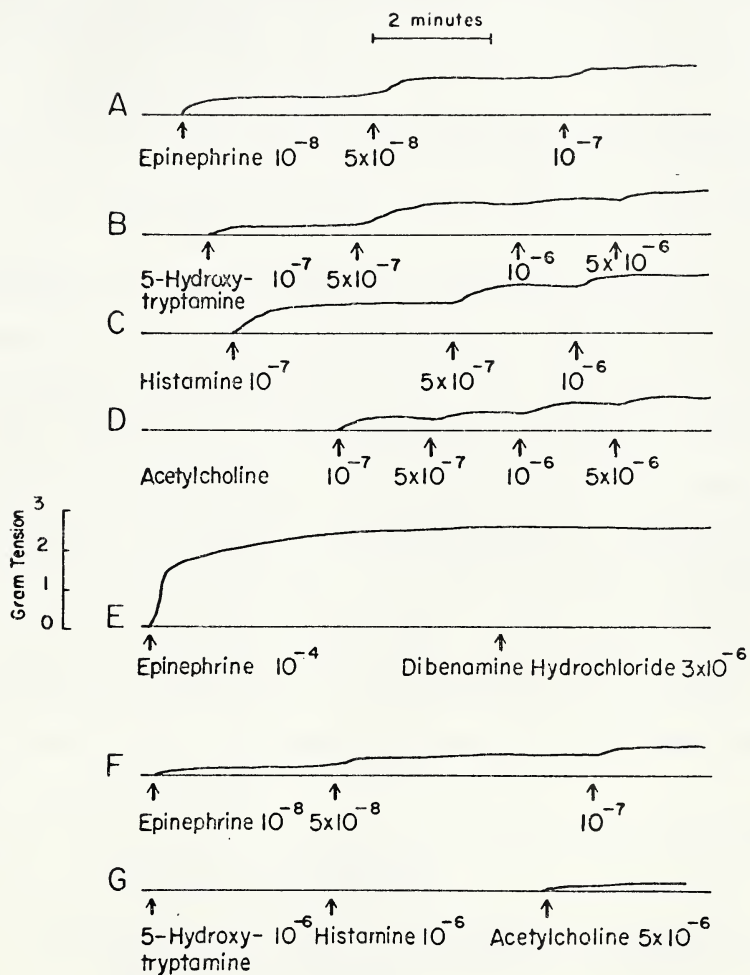


Fig. 12. The Responses of Rabbit Aortic Strips to Various Drugs in Labeling Studies with Procedure I.



ii. Determination of the Degree of Protection of the  $\alpha$ -Adrenergic Receptors

Furchgott (1954) arbitrarily defined the sensitivity of a rabbit aortic strip to a drug as follows:

$$\text{Sensitivity (S)} = T/D$$

where T = Millimeter rise of lever during a small contraction

D = Concentration (g/ml) of drug used to produce the rise (T)

He defined the degree of protection of the  $\alpha$ -adrenergic receptors of rabbit aortic strips as the per cent ratio of sensitivity to epinephrine after exposure of the tissue to epinephrine ( $1 \times 10^{-4}$ ) and dibenamine hydrochloride ( $3 \times 10^{-6}$ ) to the sensitivity to epinephrine before exposure to these drugs. A small contraction was defined as a contraction which was less than 10% of the maximal attainable contraction. Following exposure to epinephrine ( $1 \times 10^{-4}$ ) and dibenamine hydrochloride ( $3 \times 10^{-6}$ ) the first measurable contraction is referred to as a small contraction. In our studies we have calculated the degree of protection by a modification of this method. Thus the degree of protection was calculated by means of the following equation:

$$P = R'/R \times 100$$

where P (the degree of protection) = percentage of original response to epinephrine

R = Tension (g) developed by strips to a dose of epinephrine prior to epinephrine ( $1 \times 10^{-4}$ ) and dibenamine ( $3 \times 10^{-6}$ )

R' = Tension (g) developed by strips to the same dose of epinephrine after epinephrine ( $1 \times 10^{-4}$ ) and dibenamine ( $3 \times 10^{-6}$ )

The degree of protection was determined in the above manner with several doses of epinephrine and an average value was obtained.





### iii. Radioactivity Determination

#### (a) Determination of Balance Point and Counting Efficiency of Liquid Scintillation Counter

A standard unquenched  $^{14}\text{C}$ -toluene sample was used to determine the balance point of the Nuclear Chicago Liquid Scintillation System 724-725 by adjusting the high voltage of the Data and Gate photomultiplier tubes to obtain a maximal counting rate for the standard sample.  $\beta$ -Particles of different energy levels are separated by means of discriminators in the liquid scintillation counter into suitable regions called channels. With any radioactive substance emitting  $\beta$ -particles, the ratio of the observed count rate in two channels ( $L_3$ - $L_4$ ) and ( $L_3$ - $L_5$ ) varies in a way which is a measure of the efficiency with which the radioactive sample is counted by the apparatus as outlined below: A series of standard  $^{14}\text{C}$ -toluene sample solutions quenched to different degrees was counted in channels  $L_3$ - $L_4$  and  $L_3$ - $L_5$ . These count rates are designated as counts per minute (c/min), and dis/min refers to the number of disintegrations per minute in the standard samples. The ratio of counts observed in channel  $L_3$ - $L_4$  to the counts observed in channel  $L_3$ - $L_5$  is designated the channel ratio of the sample. A calibration curve was constructed by plotting the percentage efficiency of counting ( $\text{c/min} \div \text{dis/min} \times 100$ ) of each quenched standard sample against the channel ratio determined for the particular sample (Fig. 13). From Fig. 13 the c/min observed for any radioactive sample could be corrected into dis/min from the observed channel ratio of the particular sample. A similar procedure for the determination of balance point and counting efficiency was carried out in the Nuclear Chicago Unilux model 6850 liquid scintillation counter.

Each of the samples was counted for 40 min, and the background



was counted for 80 min. These time periods were calculated to be long enough to bring the percentage standard error for the corrected count down to  $\pm 2\%$ .

iv. Distribution of Radioactivity in Lipid Extracts and Lipid-Free Residues of Experimental and Control Aortic Strips

Strips labeled with  $^{14}\text{C}$ -dibenamine hydrochloride were weighed, dried from the frozen state and reweighed. The dried strips were placed in Potter-Elvehjem homogenizers (capacity 5 ml), and homogenized at room temperature with 20 ml of chloroform:methanol (2:1 v/v) per g wet weight of tissue (Folch, Lees & Sloane Stanley, 1957). In order to facilitate sedimentation of suspended material, the specific gravity of the homogenate was lowered by the addition of 0.2 ml of methanol per 1 ml of chloroform:methanol mixture. The homogenate was centrifuged at 2,000 r.p.m. for 20 min and the clear supernatant removed by means of a Pasteur pipette. The residue was re-extracted with chloroform:methanol and the supernatant fluids combined. Chloroform was added to the combined supernatant to adjust the chloroform:methanol ratio to 2:1. A portion of the chloroform:methanol extract (0.8 or 1.0 ml) was transferred to a counting vial of 20 ml capacity and the solvent removed by gentle aeration. The residue was dissolved in scintillation solution (18 ml) which was prepared by dissolving 6 g of 2,5-diphenyl oxazole (PPO) and 0.1 g of 2-p-phenylene-bis(5-phenyl oxazole) (POPOP) in 1 liter of reagent grade toluene. The lipid-free residue in the homogenizer was dissolved at  $70^\circ$  in 1 ml 5N KOH/50 mg original dry tissue; 0.2 ml aliquots of the alkaline solution were added to a mixture of methanol (5.3 ml) and scintillation solution (12.5 ml) prepared as described above. The



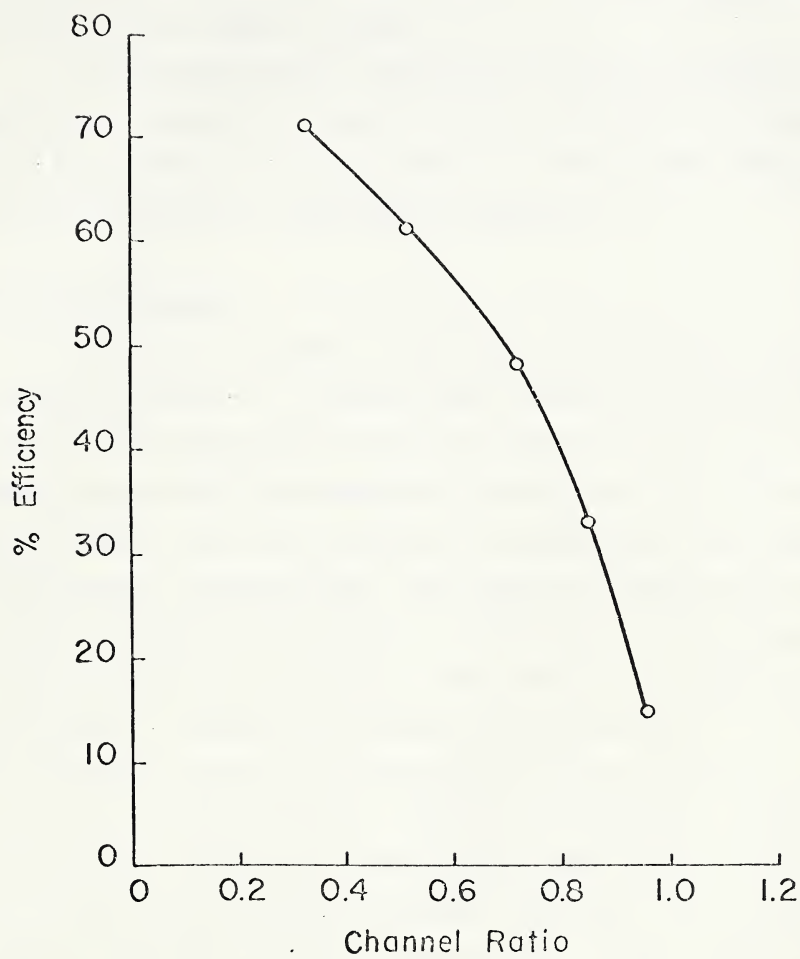


Fig. 13. Carbon-14 Channel Ratio Quench Correction Curve.



radioactivity of lipid extracts and lipid-free residues was determined in a liquid scintillation counter. The counting efficiency of lipid extracts was found to be in the range of 60-75%, while that of lipid-free residues was found to be in the range of 40-50%.

To determine whether significant differences existed between the radioactivity present in the lipid extracts and lipid-free residues of control and experimental strips the unpaired t-test was used (Ferguson, 1959). Details of the method are described in Appendix I.

### Results and Discussion

The results obtained in nine experiments with labeling procedure I are recorded in Table II. A significant increment in radioactivity ( $P < 0.05$ ) was noted in the experimental strips as compared to the control strips. This increment in radioactivity represents that portion of the  $^{14}\text{C}$ -dibenamine hydrochloride combined with sites protected by epinephrine, which might be in large part  $\alpha$ -adrenergic receptor sites. The amount of radioactivity associated with the lipid-free residue was found to be 63% of the total radioactivity bound to the tissue, whereas only 37% of the total radioactivity was located in the lipid extract. A significant increment in radioactivity ( $P < 0.05$ ) was noted in the lipid-free residues of experimental strips as compared to the lipid-free residues of control strips. However, no significant difference ( $P > 0.05$ ) in radioactivity was found on comparing the lipid extracts of experimental and control strips labeled by this procedure. The above observations differ from the report of Dikstein, Silber & Sulman (1963). These workers showed that the lipid extract obtained from rabbit aortic strips labeled with  $^3\text{H}$ -dibenamine hydrochloride contains at least 80% of the total radioactivity bound to the tissue. Moreover, they presented evidence to show that epinephrine prevents the uptake of  $^3\text{H}$ -dibenamine hydrochloride into the lipid extract. For this reason Dikstein & Sulman (1965) completely ignored the radioactivity which is associated with the non-lipid residue. Our findings clearly indicated that the radioactivity in the lipid-free residue cannot be overlooked in the search





TABLE II. THE DISINTEGRATIONS PER MINUTE DETECTED IN THE LIPID EXTRACTS AND LIPID-FREE RESIDUES OF EXPERIMENTAL AND CONTROL RABBIT AORTIC STRIPS LABELED BY PROCEDURE I.

Remaining Sensitivity to Adrenaline (% of original)	Dry Weight of Aortic Strip (mg)		Dis/min in Lipid-Free Residue/mg Dry Weight of Strip		Dis/min in Lipid Extract /mg Dry Weight of Strip		Total dis/min/mg of Dry Weight of Strip	
	Control Strip	Experimental Strip	Control Strip	Experimental Strip	Control Strip	Experimental Strip	Control Strip	Experimental Strip
62	12.6	13.1	93	127	64	75	157	202
71	13.6	16.3	86	92	38	39	124	131
67	13.0	12.6	83	137	32	56	115	193
50	17.5	14.9	51	84	39	56	90	140
29	15.3	13.7	61	76	48	59	109	135
50	15.1	14.7	78	79	48	49	126	128
70	15.8	13.1	87	79	49	44	136	124
60	13.9	14.0	81	71	55	48	136	119
30	15.8	16.6	63	91	35	53	98	144
Mean $\pm$ S.D.	14.7 $\pm$ 1.5	14.3 $\pm$ 1.3	76 $\pm$ 22	93 $\pm$ 22	45 $\pm$ 10	53 $\pm$ 10	121 $\pm$ 20	146 $\pm$ 28
P Value	<0.05		>0.05		<0.05		<0.05	

The total dis/min/mg dry weight of strip is the sum of the dis/min/mg of the lipid extract and lipid-free residue.



for the dibenamine- $\alpha$ -adrenergic receptor complex.

Of considerable interest was the finding that the control strips contained a large amount of radioactivity ( $121 \pm 20$  dis/min/mg dry weight of strip). This finding is hard to reconcile with theoretical expectations (Gaddum, 1962). It will be recalled that the receptors of epinephrine, 5-hydroxytryptamine, histamine and acetylcholine in the control strips were irreversibly blocked by unlabeled dibenamine hydrochloride ( $3 \times 10^{-6}$ ). Consequently it was expected that following exposure of strips to unlabeled dibenamine hydrochloride only negligible amounts of  $^{14}\text{C}$ -dibenamine hydrochloride would be taken up by the strips. Clearly this was not the case and for this reason it was necessary to carefully re-investigate the above labeling procedure proposed by Gaddum (1962). The results of this study will be presented in Chapter III.

### C. An Alternative Procedure for Labeling the $\alpha$ -Adrenergic Receptors (Procedure II)

#### Introduction

In view of the unexpectedly high radioactivity associated with control strips labeled by procedure I, it was decided to study the possibility of utilizing a simpler labeling procedure described by Dikstein & Sulman (1965). The experimental design was as follows: A rabbit aortic strip (Fig. 14a) was treated with epinephrine to protect the adrenergic receptors (Fig. 14b) and  $^{14}\text{C}$ -dibenamine hydrochloride added to react with the 5-hydroxytryptamine, histamine, and acetylcholine receptors (Fig. 14c). The tissue was then washed with Krebs's bicarbonate solution to remove epinephrine and loosely bound  $^{14}\text{C}$ -dibenamine hydrochloride (Fig. 14d). A strip treated in this manner was designated the experimental strip. As a control the experiment was repeated with the omission of the protecting



dose of epinephrine in the first step (Fig. 14a',c',d '). This labeling procedure has been designated labeling procedure II.

Due to the fact that four receptors in the control strips were blocked by  $^{14}\text{C}$ -dibenamine hydrochloride while in the experimental strips only the 5-hydroxytryptamine, histamine, and acetylcholine receptors were blocked by this agent, it was reasonable to assume that the control strips would contain greater radioactivity than the experimental strips. Moreover, this difference in radioactivity represents that portion of the  $^{14}\text{C}$ -dibenamine hydrochloride combined with sites in the control strips that are protected by epinephrine in the experimental strips. These sites might be in large part  $\alpha$ -adrenergic receptors. A series of experiments was then carried out to see that this was in fact the case.

In view of the claim of Dikstein & Sulman (1965) that cephalin is the tissue receptor for epinephrine the amount of radioactivity in both lipid extract and lipid-free residue of the aortic strips was determined.

### Experimental

Rabbit aortic strips were mounted as described in labeling procedure I. The response of the aortic strips to several concentrations of epinephrine was recorded (Fig. 15A). The strips were then treated with epinephrine ( $1 \times 10^{-4}$ ) for 5 min, and  $^{14}\text{C}$ -dibenamine hydrochloride ( $3 \times 10^{-6}$ ) added (Fig. 15B). After a further 20 min period the strips were washed three times with epinephrine ( $1 \times 10^{-4}$ ) and three times with bath fluid. The strips were washed with bath fluid at 15 min intervals until they relaxed completely and were shown to retain considerable responsiveness to epinephrine (Fig. 15C). Strips treated as above have been designated experimental



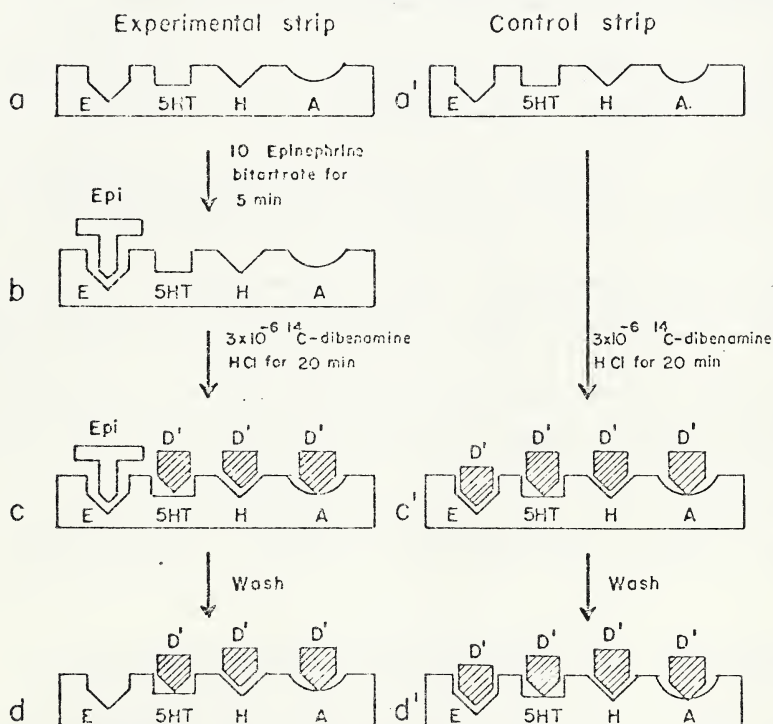


Fig. 14. Schematic Representation of Labeling Procedure II.

E,  $\alpha$ -adrenergic receptor; 5HT, 5-hydroxytryptamine receptor; H, histamine receptor; A, acetylcholine receptor;  $D'$ ,  $^{14}$ C-dibenamine hydrochloride; Epi, epinephrine.





strips. As controls the experiments were repeated with the omission of the protecting dose of epinephrine ( $1 \times 10^{-4}$ ) in the first step. The radioactivity associated with the strips was determined as described for procedure I.

### Results and Discussion

The results obtained in seven experiments with labeling procedure II are recorded in Table III. In accordance with expectation a significant increment in radioactivity ( $P < 0.05$ ) was noted in control strips as compared to experimental strips. A significant increment in radioactivity ( $P < 0.05$ ) was also noted in lipid extracts of control strips as compared to lipid extracts of experimental strips. These results are in agreement with those obtained by Dikstein & Sulman (1965) who used an analogous procedure. However, it is clear that the lipid-free residue cannot be ignored in the search for a dibenamine- $\alpha$ -adrenergic receptor complex because of the significant increment in radioactivity ( $P < 0.05$ ) in the lipid-free residue of the control strips, as compared to the lipid-free residue of experimental strips.

Of considerable interest was the finding that only 20-23% of the total radioactivity of the strips was associated with the lipid extracts in the labeling procedure II. This finding differs from the report of Dikstein, Silber & Sulman (1963) who, using a similar labeling procedure, located at least 80% of the bound radioactivity in the lipid extract. Dikstein & Sulman (1965) extracted the lipids of aortic strips by boiling the tissue with chloroform:methanol (2:1) for 2 min. In our experiments the lipids of aortic strips were extracted according to the method of Folch, Lees & Sloane



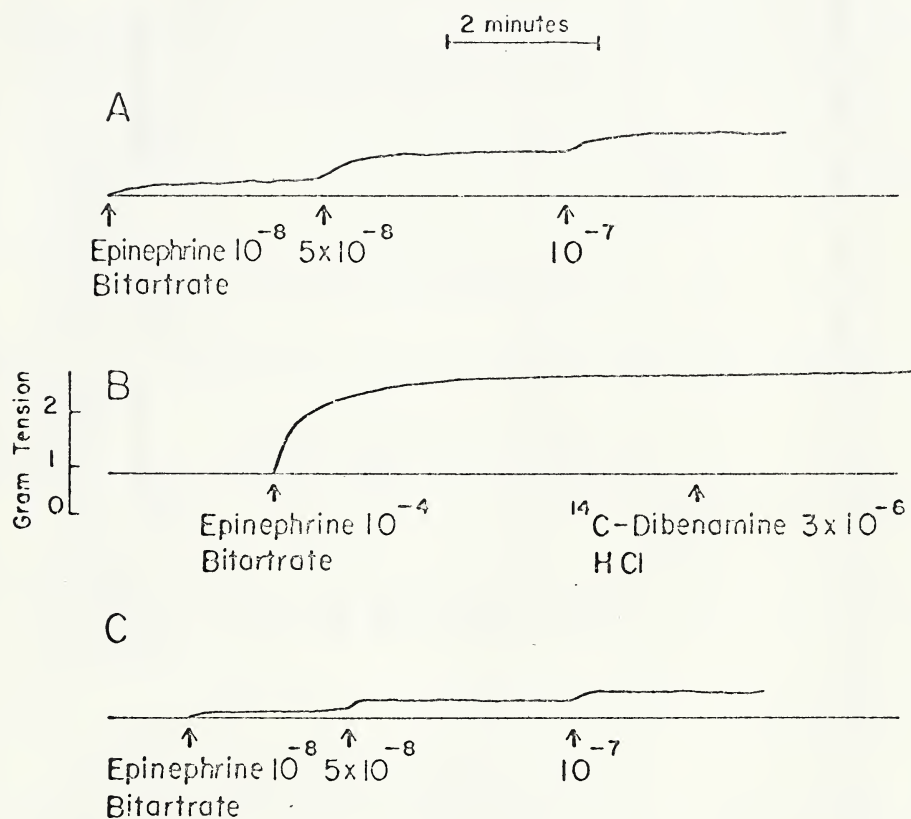


Fig. 15. The Response of Rabbit Aortic Strips to Epinephrine in Labeling Studies with Procedure II.



TABLE III. THE DISINTEGRATIONS PER MINUTE DETECTED IN THE LIPID EXTRACTS AND LIPID-FREE RESIDUES OF EXPERIMENTAL AND CONTROL RABBIT AORTIC STRIPS LABELED BY PROCEDURE II.

Remaining Sensitivity to Adrenaline (% of original)	Dry Weight of Aortic Strip (mg)		Dis/min in Lipid-Free Residue/mg Dry Weight of Strip		Dis/min in Lipid Extract /mg Dry Weight of Strip		Total dis/min/mg of Dry Weight of Strip	
	Control Strip	Experimental Strip	Control Strip	Experimental Strip	Control Strip	Experimental Strip	Control Strip	Experimental Strip
30	18.6	18.7	121	83	21	14	142	97
50	12.8	14.8	88	58	27	11	115	69
25	21.2	18.6	101	76	36	18	137	94
15	15.3	16.3	105	106	32	31	137	137
25	21.0	18.4	79	77	36	23	115	100
30	17.0	22.0	111	69	27	19	138	88
25	15.6	15.7	114	91	35	24	148	115
Mean $\pm$ S.D.	17.4 $\pm$ 2.9	17.8 $\pm$ 2.3	103 $\pm$ 14	80 $\pm$ 14	31 $\pm$ 5	20 $\pm$ 6	133 $\pm$ 12	100 $\pm$ 20
P Value	<0.05		<0.05		<0.05		<0.05	

The total dis/min/mg dry weight of strip is the sum of the dis/min/mg of the lipid extract and lipid-free residue.



Stanley (1957) by homogenizing the tissue twice with chloroform:methanol (2:1) at room temperature. In order to check whether there was incomplete removal of lipid-bound  $^{14}\text{C}$ -dibenamine in our experiments, the residue which was extracted with chloroform:methanol at room temperature was re-extracted twice with hot chloroform:methanol (2:1). However, examination of this extract by thin-layer chromatography showed that there was no lipid present. Moreover, no radioactivity was detected in this extract. The smaller amount of radioactivity located in the lipid extracts in our experiments as compared to those of Dikstein, Silber & Sulman (1963) cannot be explained by incomplete extraction.

#### D. Thin-Layer Chromatography of the Lipid Extracts of Aortic Strips

##### Introduction

Experimental and control strips labeled by Procedures I and II were subjected to chemical fractionation with the object of isolating a  $^{14}\text{C}$ -dibenamine- $\alpha$ -adrenergic receptor complex. This complex should possess the following characteristics: it should be a substance isolated from the experimental strips with a higher  $^{14}\text{C}$ -content than a corresponding substance isolated from the control strips (procedure I), and for this reason should be identifiable. Conversely, with labeling procedure II, it should be a substance isolated from the control strips with a higher  $^{14}\text{C}$ -content than a corresponding substance isolated from the experimental strips. Dikstein & Sulman (1965) have fractionated the lipid extracts obtained from aortic strips labeled by procedure I on thin-layer chromatograms. By this method they showed that the amount of radioactivity associated with





cephalin obtained from experimental strips was greater than that obtained from control strips. The converse was found to be the case using labeling procedure II. For this reason they suggested that cephalin might be the tissue receptor for epinephrine. In view of this observation, it was of interest to study the distribution of radioactivity in the lipid components of the lipid extracts by means of thin-layer chromatography and to compare the results with those reported by Dikstein & Sulman (1965).

## Experimental

### i. Purification of Lipid Extracts

Folch, Lees & Sloane Stanley (1957) have described a simple method for the extraction of tissue lipids in pure form. The method consists of homogenizing the tissue with chloroform:methanol (2:1) at room temperature and washing the chloroform:methanol extract with 0.2 volumes of 0.88% KCl solution to remove non-lipid material. Thus, following the above procedure, a part of the chloroform:methanol extract from the labeled aortic strips was mixed thoroughly with 0.2 volumes of 0.88% KCl solution, and the mixture centrifuged at low speed for 10 min in order to separate the two phases. Most of the upper phase, which constituted 40% of the total volume of the system, was removed by means of a Pasteur pipette. The remaining portion of the upper phase was removed in the following manner: A solution containing the same solvent composition as the upper phase, viz., chloroform:methanol:water (3:48:47 v/v) was prepared and added carefully to the lower phase. The upper phase was then removed by means of a Pasteur pipette. This procedure was repeated twice and the washed lower phases from three experiments combined. A few drops of methanol were added to the combined



lower phases and the resulting solution concentrated to 0.3 ml by removing the solvent under reduced pressure at room temperature.

## ii. Preparation of Chromatoplates

Chromatoplates (20 cm x 20 cm) were prepared by a modification of the procedure described by Skipski, Peterson & Barclay (1962). A slurry was made by mixing 10 g of silica gel G and 27 ml of 0.01 M sodium acetate solution in a mortar. The slurry was applied to the carrier glass plates on the spreading template with the aid of a coating applicator (Desaga) adjusted to a thickness of 250  $\mu$ . Plates were allowed to dry on the template at room temperature for 25 min and then activated at 90° for 45 min prior to the application of samples.

## iii. Application of Samples

The following substances were used as standards: sphingomyelin (purified from beef brain, Sigma Chemical); phosphatidyl-L-serine (fraction III of Folch, from bovine brain, grade II, Sigma Chemical); L- $\alpha$ -cephalin ( $\beta,\gamma$ -dipalmitoyl) synthetic (lyso-free, B grade, Calbiochem); phosphatidyl-ethanolamine (fraction V of Folch, from bovine brain, Grade II, Sigma Chemical). The samples in chloroform:methanol (2:1) were applied with micro-pipettes at a distance of 2.5 cm from the bottom of the carrier plate. The amount of standard compound applied was in the range of 20-40  $\mu$ g. The concentrated lipid extracts from aortic strips were applied in quantities ranging from 100 to 200  $\mu$ l. The following substances were applied to each chromatoplate: (1) purified lipid extracts of unlabeled rabbit aortic strips; (2) purified lipid extracts of experimental strips; (3) purified



lipid extracts of control strips; (4)  $^{14}\text{C}$ -dibenamine hydrochloride; (5) sphingomyelin; (6) phosphatidyl-L-serine; (7) L- $\alpha$ -cephalin or phosphatidylethanolamine; (8) a mixture of (5), (6) and (7). The chromatographic chambers were lined on three sides with Whatman no. 1 paper. Sixty minutes prior to insertion of the chromatoplates, developing solvent was added to wet the paper and for subsequent development of the chromatoplate. The solvent system consisted of a mixture of chloroform, methanol and water in the proportions 75:22:3 by volume (Dikstein & Sulman, 1965). The chromatogram was allowed to develop at room temperature for 80 to 90 min by which time the solvent had traveled a distance of 14 cm.

#### iv. Determination of Radioactivity Associated with Individual Lipid Components

The developed plates were dried in air at room temperature and then transferred to a glass chamber saturated with iodine vapor. After 20 min, individual compounds were detected as brown spots and marked. Marked segments of the chromatogram were scraped into small glass chromatographic columns after decolorization of the brown spots upon exposure to the atmosphere. The columns were eluted three times with 1 ml of chloroform: methanol (2:1) and the eluate evaporated to dryness in a counting vial by passing a stream of air over the surface of the solvent. Counting solution (18 ml) was added to dissolve the residue and the radioactivity determined. The recovery of radioactivity based on experiments in which known amounts of pure  $^{14}\text{C}$ -dibenamine hydrochloride were applied to the plates was found to be approximately 65%.





## Results and Discussion

A typical thin-layer chromatogram showing the separation of the lipid components of rabbit aortic tissue is presented in Fig. 16. The distribution of radioactivity in the lipid components is recorded in Table IV (labeling procedure I) and Table V (labeling procedure II). The bulk of the radioactivity was detected in the solvent front, which was the position to which  $^{14}\text{C}$ -dibenamine hydrochloride moved in this solvent system. The amount of radioactivity associated with the cephalin fraction represented less than 3% of the total radioactivity present in the aortic strip. Moreover, no significant difference was discerned in the amount of radioactivity in the cephalin fraction of experimental and control strips. This finding is contrary to that of Dikstein & Sulman (1965). The latter workers, using a procedure analogous to procedure I in this study, reported that the radioactivity in the cephalin fraction of experimental strips was greater than in control strips. Conversely, they detected more radioactivity in the cephalin fraction of control strips than in experimental strips, by a procedure analogous to procedure II in this study. Thus we are unable to substantiate the findings of Dikstein & Sulman regarding the radioactivity associated with the cephalin fraction. It is curious that Dikstein & Sulman did not consider the fact that a complex between dibenamine and cephalin would in all likelihood have a different  $R_f$  value than cephalin. For this reason even if we had been able to duplicate their results we would not be able to claim that cephalin was a receptor for epinephrine. The nature of the major radioactive component in our thin-layer chromatograms ( $R_f$  range 0.84 - 1.00) required further investigation, since it was not possible to unequivocally decide whether it consisted of free dibenamine or a complex between dibenamine





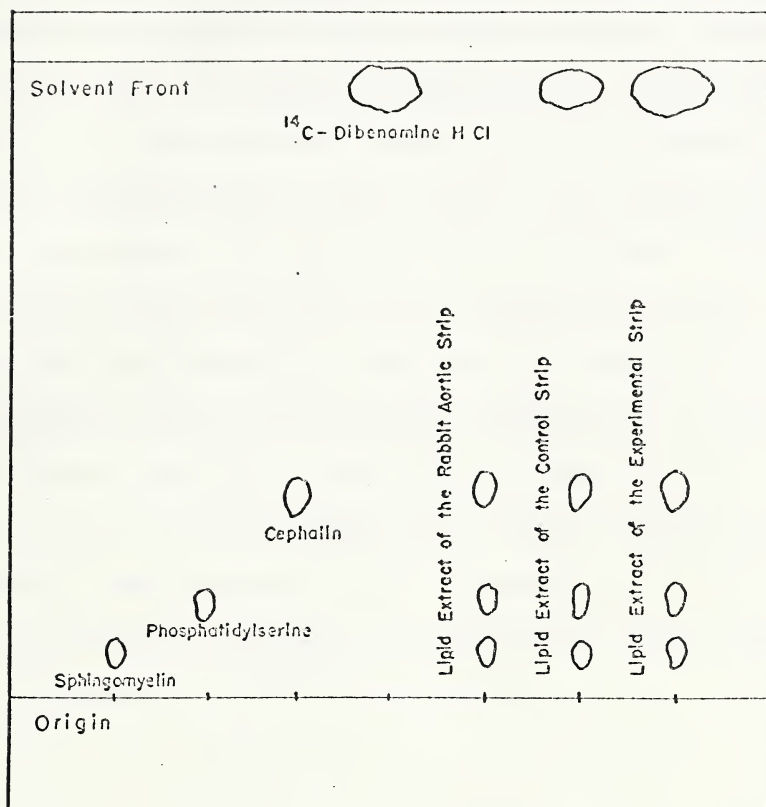


Fig. 16. A Thin-Layer Chromatogram of Different Reference Phospholipids,  $^{14}\text{C}$ -Dibenamine Hydrochloride, and Lipid Extracts of Rabbit Aortic Strips.



and a lipid component. However, in view of the fact that only 20% of the radioactivity was located in the lipid extract and that we could not confirm the results of Dikstein & Sulman, we decided to defer further study of the lipid fraction to a later stage of this thesis.

In seeking to understand the difference between the results obtained by us and those obtained by Dikstein & Sulman, the following facts are of interest: (1) Dikstein & Sulman have not studied the degree of protection of the  $\alpha$ -adrenergic receptor afforded by epinephrine ( $1 \times 10^{-4}$ ) against dibenamine hydrochloride ( $1 \times 10^{-5}$ ), the concentrations used in their experiments. In view of the report of Furchgott (1954) that no protection or doubtful protection of the  $\alpha$ -adrenergic receptor is obtained under these conditions, it is likely that the concentrations of epinephrine and dibenamine hydrochloride used were inappropriate. Moreover, the aortic strips used by Dikstein & Sulman were not subjected to any loading tension. (2) Tritium is able to exchange with the labile hydrogen atoms of the solvent or tissue components, and it is possible that a part of the radioactivity detected in the lipid extracts by these workers might have been due to exchange of tritium for hydrogen.



TABLE IV. THE DISINTEGRATIONS PER MINUTE DETECTED IN THE COMPONENTS OF LIPID EXTRACTS OF EXPERIMENTAL AND CONTROL RABBIT AORTIC STRIPS LABELED BY PROCEDURE I.

R <sub>f</sub> Value	17.5 µg <sup>14</sup> C-dibenamine HCl	Total dis/min per Segment of Chromatogram							
		Experiment 1		Experiment 2		Experiment 3		Experiment 4	
		Control	Experi- mental	Control	Experi- mental	Control	Experi- mental	Control	Experi- mental
		Strip	Strip	Strip	Strip	Strip	Strip	Strip	Strip
0-0.04	32	0	4	1	0	0	2	0	1
0.04-0.16*	25	4	5	0	0	1	0	0	1
0.16-0.34†	59	3	4	1	1	0	2	0	0
0.34-0.63‡	94	3	3	2	6	0	6	0	0
0.63-0.87	215	2	5	8	7	2	1	2	0
0.87-1	20,173	55	70	37	90	39	39	45	46
Total	20,598	67	91	49	104	42	50	47	48

\* Range for sphingomyelin.

† Range for phosphatidyl serine.

‡ Range for cephalin.



TABLE V. THE DISINTEGRATIONS PER MINUTE DETECTED IN THE COMPONENTS OF LIPID EXTRACTS OF EXPERIMENTAL AND CONTROL RABBIT AORTIC STRIPS LABELED BY PROCEDURE II.

R <sub>f</sub> Value	17.5 µg <sup>14</sup> C-dibenamine HCl	Total dis/min per Segment of Chromatogram							
		Experiment 1		Experiment 2		Experiment 3		Experiment 4	
		Control Strip	Experi-mental Strip	Control Strip	Experi-mental Strip	Control Strip	Experi-mental Strip	Control Strip	Experi-mental Strip
0-0.03	32	0	0	0	0	1	0	0	0
0.03-0.18*	25	8	0	0	0	1	0	4	0
0.18-0.35†	59	2	0	0	1	5	2	5	0
0.35-0.60‡	94	3	4	2	3	4	6	7	6
0.60-0.84	215	3	4	2	2	2	3	6	7
0.84-1.00	20,173	60	40	95	51	101	59	90	58
Total	20,598	76	48	99	57	114	70	112	71

\* Range for sphingomyelin.

† Range for phosphatidyl serine.

‡ Range for cephalin.





CHAPTER III    DETAILED INVESTIGATION OF THE STEPS INVOLVED  
IN THE LABELING PROCEDURES



A. Removal of Loosely Bound  $^{14}\text{C}$ -Dibenamine Hydrochloride from Rabbit Aortic Strips

Introduction

In Chapter II of this thesis experiments were described showing that the lipid extracts of rabbit aortic strips labeled by procedure I contained 37% of the total radioactivity bound to the tissue, while lipid extracts from strips labeled by procedure II contained only 20-23% of the total radioactivity. Dikstein & Sulman (1965) on the other hand located at least 80% of the total radioactivity in the lipid extracts of rabbit aortic strips labeled by a procedure analogous to procedure II. Since different methods were used for washing the tissues after labeling, it was possible that loosely bound radioactive dibenamine hydrochloride was incompletely removed in the above labeling procedures. It was therefore likely that loosely bound radioactive dibenamine hydrochloride contributed to the radioactivity in the lipid extracts of aortic strips and accounted for the variation observed in the lipid extracts obtained in different labeling procedures. For the above reason experiments have been carried out to establish an adequate procedure for removing loosely bound  $^{14}\text{C}$ -dibenamine hydrochloride prior to chemical fractionation of aortic tissue. Furthermore, it was of interest to compare the removal of  $^{14}\text{C}$ -dibenamine hydrochloride from strips treated directly with this agent as in procedure II and the removal of  $^{14}\text{C}$ -dibenamine hydrochloride from strips pretreated with unlabeled dibenamine hydrochloride as in procedure I.

After establishing an adequate procedure for removing loosely bound radioactivity from aortic strips, we next carried out experiments to



determine whether  $^{14}\text{C}$ -dibenamine hydrochloride, retained by aortic strips after washing with Krebs's bicarbonate solution, was exchangeable with unlabeled dibenamine hydrochloride in the bath fluid.

### Experimental

(a) Rabbit aortic strips were mounted as single strips in organ baths as shown in Fig. 9B. The response of four aortic strips to epinephrine was recorded and the strips then exposed to  $^{14}\text{C}$ -dibenamine hydrochloride ( $3 \times 10^{-6}$ ) for 20 min. One strip was removed from an organ bath without washing and the remaining three strips were washed with Krebs's bicarbonate solution at 15 min intervals. A second strip was removed from an organ bath after 1 hr, a third strip after 3 hr and a fourth strip after 6 hr. The radioactivity in the lipid extracts and lipid-free residues of the strips was then determined.

(b) Rabbit aortic strips were exposed to unlabeled dibenamine hydrochloride ( $3 \times 10^{-6}$ ) for 20 min. After one wash, strips were exposed to  $^{14}\text{C}$ -dibenamine hydrochloride ( $3 \times 10^{-6}$ ) for 20 min. One strip was removed from an organ bath without washing and the remaining three strips were washed with Krebs's bicarbonate solution at 15 min intervals. A second strip was removed from an organ bath after 1 hr, a third strip after 3 hr and a fourth strip after 6 hr. The radioactivity in the lipid extracts and lipid-free residues of the strips was determined.

(c) The response of four aortic strips to epinephrine was recorded. The strips were exposed to  $^{14}\text{C}$ -dibenamine hydrochloride ( $3 \times 10^{-6}$ ) for 20 min and the strips then washed with Krebs's bicarbonate solution, containing unlabeled dibenamine hydrochloride ( $3 \times 10^{-6}$ ) at 15 min intervals.



Because of the instability of dibenamine hydrochloride, fresh solutions of this agent in Krebs's bicarbonate solution were made up at 15 min intervals. One strip was removed from an organ bath after 1 hr and a second strip after 4 hr; the radioactivity in the lipid extracts and lipid-free residues of the strips was determined. As a control the above experiment was repeated with the omission of unlabeled dibenamine hydrochloride ( $3 \times 10^{-6}$ ) from the Krebs's bicarbonate wash fluid.

### Results and Discussion

After exposing aortic strips to  $^{14}\text{C}$ -dibenamine hydrochloride ( $3 \times 10^{-6}$ ), approximately 50% of the radioactivity taken up could be removed by washing the strips for 3 hr at 15 min intervals (Fig. 17). Extending the period of washing to 6 hr did not result in a further significant removal of radioactivity. The radioactivity removed by washing may be associated with loosely bound dibenamine hydrochloride and/or its transformation products. Of the  $^{14}\text{C}$ -dibenamine hydrochloride taken up by the lipid-free residue, 31% was removable by washing, whereas 77% of the radioactivity associated with the lipid fraction was removable by washing. It follows that if a tissue is not adequately washed after labeling with  $^{14}\text{C}$ -dibenamine hydrochloride, a high percentage of radioactivity will be found in the lipid fraction. Differences in washing procedures might therefore account for failure to observe a similar percentage of radioactivity associated with lipid extracts in different labeling procedures. Dikstein & Sulman washed their labeled aortic strips with Krebs's bicarbonate solution at 10 min intervals for 50 min and added a final wash after a further 1 hr period. It is probable that incomplete removal of loosely bound  $^3\text{H}$ -dibenamine hydrochloride





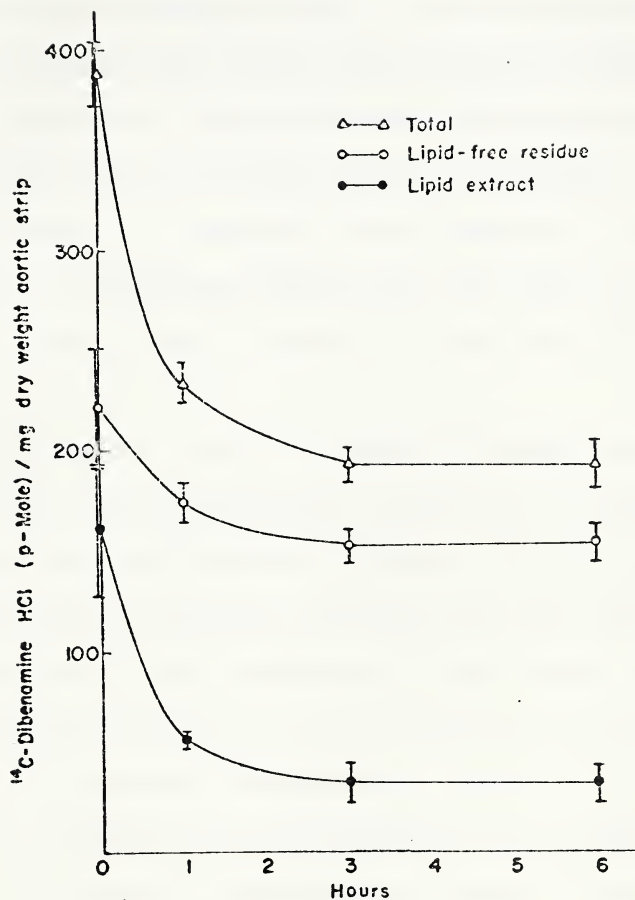


Fig. 17. Removal of Loosely Bound  $^{14}\text{C}$ -Dibenamine Hydrochloride from Rabbit Aortic Strips. Vertical lines represent  $\pm$  S.D. of the mean. Each point represents the mean of 2 to 4 independent observations.



under these conditions could account, at least partially, for the high recovery of radioactivity in their lipid extracts. In our former experiments, strips labeled by procedure I were washed for 20 min while in procedure II the period of washing was  $1\frac{1}{2}$  hr. The fact that removal of loosely bound radioactivity from labeled strips requires at least 3 hr of washing probably explains the difference between the percentage radioactivity associated with lipid extracts of strips labeled by procedure I (37%) and by procedure II (20-23%). In subsequent studies described in this thesis, strips labeled with  $^{14}\text{C}$ -dibenamine hydrochloride were washed for 3 hr at 15 min intervals to ensure complete removal of loosely bound  $^{14}\text{C}$ -dibenamine hydrochloride.

Initial exposure of strips to unlabeled dibenamine hydrochloride ( $3 \times 10^{-6}$ ) for 20 min lowered the subsequent uptake of  $^{14}\text{C}$ -dibenamine hydrochloride from 380 to 300 dis/min/mg dry weight of strip (Fig. 18). The removal of loosely bound  $^{14}\text{C}$ -dibenamine hydrochloride from these strips, followed the same course as that observed with strips treated with  $^{14}\text{C}$ -dibenamine hydrochloride alone (Fig. 17). The results recorded in Table VI show that addition of unlabeled dibenamine hydrochloride to the wash fluid does not result in a lowered retention of radioactivity by rabbit aortic strips. Thus the  $^{14}\text{C}$ -dibenamine hydrochloride retained by the strips is not exchangeable with unlabeled dibenamine hydrochloride in the bath fluid. This finding suggests that the radioactivity retained in the rabbit aortic strips after exposure to  $^{14}\text{C}$ -dibenamine hydrochloride represents that portion of the  $^{14}\text{C}$ -dibenamine hydrochloride covalently bound to tissue components.



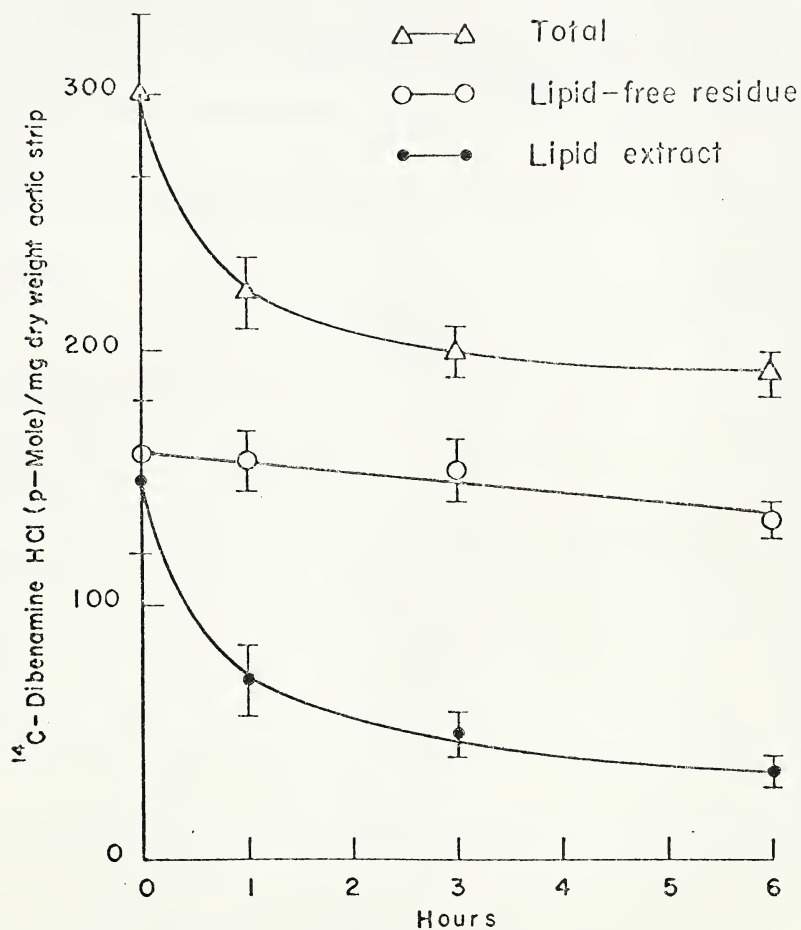


Fig. 18. Removal of Loosely Bound  $^{14}\text{C}$ -Dibenamine Hydrochloride from Rabbit Aortic Strips Pretreated with Unlabeled Dibenamine Hydrochloride.



TABLE VI. THE EFFECT OF WASHING  $^{14}\text{C}$ -DIBENAMINE  
HYDROCHLORIDE LABELED AORTIC STRIPS  
WITH UNLABELED DIBENAMINE HYDROCHLORIDE  
SOLUTION

Aortic Strip	Total dis/min/mg dry weight of strip ± S.D.	
	1 hr	4 hr
Wash with Krebs's Bicarbonate Solution	311 ± 63 (6)*	178 ± 18 (4)
Wash with $3 \times 10^{-6}$ g/ml of Dibenamine HCl	299 ± 70 (6)	217 ± 67 (4)
P Value	> 0.05	> 0.05

\*Numbers enclosed by parentheses indicate the number of experiments performed.





## B. Retention of $^{14}\text{C}$ -Labeled 2-Dibenzylaminoethanol by Rabbit Aortic Strips

### Introduction

Moran et al. (1967) have attempted to specifically label the  $\alpha$ -adrenergic receptors of rabbit vas deferens and aortic strips with  $^3\text{H}$ -labeled N-(2-bromoethyl)-N-ethyl-N-1-naphthyl-methylamine ( $^3\text{H}$ -SY.28; Fig. 5). These workers suggested that the prolonged tissue retention of radioactivity following treatment with  $^3\text{H}$ -SY.28 was not due to covalent binding but rather to the high binding capacity of tissue for the alcohol derived by hydrolysis of  $^3\text{H}$ -SY.28 at physiological pH. In our studies on the  $\alpha$ -adrenergic receptor we have utilized  $^{14}\text{C}$ -dibenamine hydrochloride which belongs to the same chemical class as  $^3\text{H}$ -SY.28, viz., the 2-halogeno-ethylamines. The success of our procedure is based on the idea that  $^{14}\text{C}$ -dibenamine hydrochloride forms a covalent bond with the  $\alpha$ -adrenergic receptor. It was therefore important to demonstrate that the prolonged retention of radioactivity observed after treatment of rabbit aortic strips with  $^{14}\text{C}$ -dibenamine hydrochloride was not due to the high binding capacity of strips for 2-dibenzylaminoethanol, the alcohol derived by hydrolysis of dibenamine hydrochloride. For this reason it was decided to prepare  $^{14}\text{C}$ -labeled 2-dibenzylaminoethanol hydrochloride by a method previously used by Gump & Nikawitz (1950) to prepare the unlabeled compound (Fig. 8). Rabbit aortic strips were exposed to  $^{14}\text{C}$ -labeled 2-dibenzylaminoethanol hydrochloride and the strips washed for various time periods. The radioactivity associated with the lipid extracts and lipid-free residues of the strips was determined.



## Experimental

### i. Synthesis of $^{14}\text{C}$ -2-Dibenzylaminoethanol Hydrochloride

Precautions connected with the handling of  $^{14}\text{C}$ -labeled compounds were observed during the preparation of  $^{14}\text{C}$ -2-dibenzylaminoethanol hydrochloride. The apparatus was set up in a hood on a special tray lined with thick absorbent paper and aluminum foil. A chilled ampoule of benzyl-7- $^{14}\text{C}$ -chloride (0.013 g in 0.7 ml benzene, specific activity 3 mc/m-mole; New England Nuclear Corp.) was opened in the hood and unlabeled benzyl chloride (0.237 g) introduced into the ampoule with a serological pipette. 2-Aminoethanol (0.122 g) was heated to 40° in a semi-micro three-necked flask equipped with an air driven stirrer and the contents of the ampoule added dropwise to the flask with the aid of a Pasteur pipette. The ampoule was rinsed with 0.7 ml of benzene and the benzene transferred to the flask. The temperature was raised to 100-110° and stirring continued for 2½ hr. A solution of 62.5% NaOH (0.12 ml) was added and the mixture stirred at 100° for 1 hr. Upon cooling the mixture was extracted with benzene (5 ml) and the benzene solution washed with water and dried (sodium sulfate). The benzene solution was transferred with a long stemmed Pasteur pipette into a micro-distillation apparatus. After removal of benzene by gentle warming in vacuo the 2-dibenzylaminoethanol distilled as a colorless oily liquid, b.p. 125-130°/0.03 mm (0.146 g; 65%). The product was dissolved in ethanol and the solution saturated with hydrogen chloride. Upon adding a few drops of ether a precipitate was obtained which was recrystallized from ethanol, affording  $^{14}\text{C}$ -labeled 2-dibenzylaminoethanol hydrochloride as white crystals (0.1 g, 57%), m.p. 171-173°. When mixed with 2-dibenzylaminoethanol hydrochloride (Aldrich Chemical Co.) it had m.p. 171-173°. The infrared spectrum



of the  $^{14}\text{C}$ -2-dibenzylaminoethanol hydrochloride was identical in all respects with that of 2-dibenzylaminoethanol hydrochloride (Fig. 19). The product was recrystallized to constant specific activity (0.32 mc/m-mole).

ii. Removal of Loosely Bound  $^{14}\text{C}$ -Dibenzylaminoethanol Hydrochloride from Rabbit Aortic Strips

The responses of eight rabbit aortic strips to several concentrations of epinephrine were recorded and the strips then exposed to  $^{14}\text{C}$ -dibenzylaminoethanol hydrochloride ( $2.8 \times 10^{-6}$ ) for 20 min. Strips 1 and 2 were removed from the organ baths without washing, and the remaining strips were washed with Krebs's bicarbonate solution at 15 min intervals. Strips 3 and 4 were removed from the organ baths after 1 hr, strips 5 and 6 after 3 hr, and strips 7 and 8 after 6 hr. The radioactivity in the lipid extracts and lipid-free residues was then determined. The results of this experiment are shown in Fig. 20.

Results and Discussion

The results (Fig. 20) show that after exposing strips to  $^{14}\text{C}$ -2-dibenzylaminoethanol hydrochloride all the radioactivity can be removed by washing for 6 hr at 15 min intervals. In a similar experiment previously carried out with  $^{14}\text{C}$ -dibenamine hydrochloride ( $3 \times 10^{-6}$ ) only 50% of the radioactivity could be removed (Fig. 17). These results clearly show that the prolonged retention of radioactivity following exposure of aortic strips to  $^{14}\text{C}$ -dibenamine hydrochloride is not due to the high binding capacity of strips for the alcohol which would be obtained upon hydrolysis of dibenamine hydrochloride, viz., 2-dibenzylaminoethanol.

It was of interest to compare the distribution of radioactivity



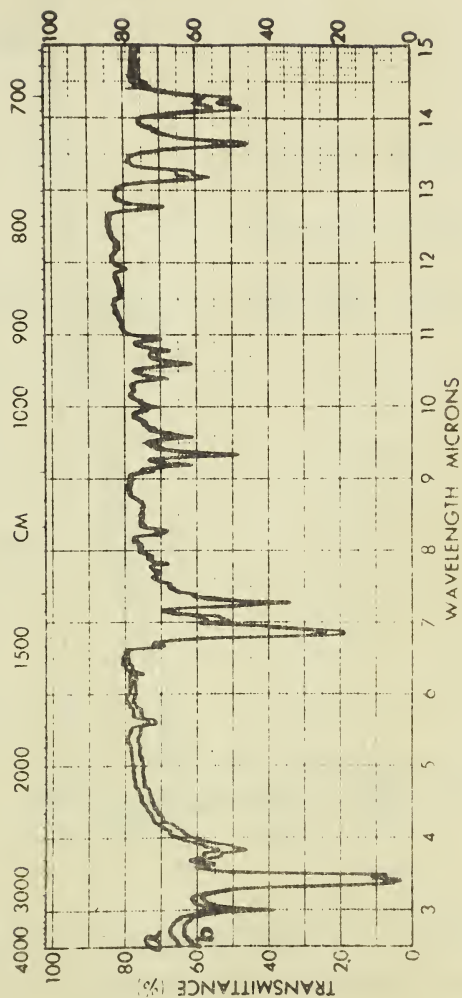


Fig. 19. Infrared Spectra in Nujol of 2-Dibenzylaminoethanol Hydrochloride. a. Unlabeled compound (Aldrich Chemicals Co.);  
b. <sup>14</sup>C-labeled compound.







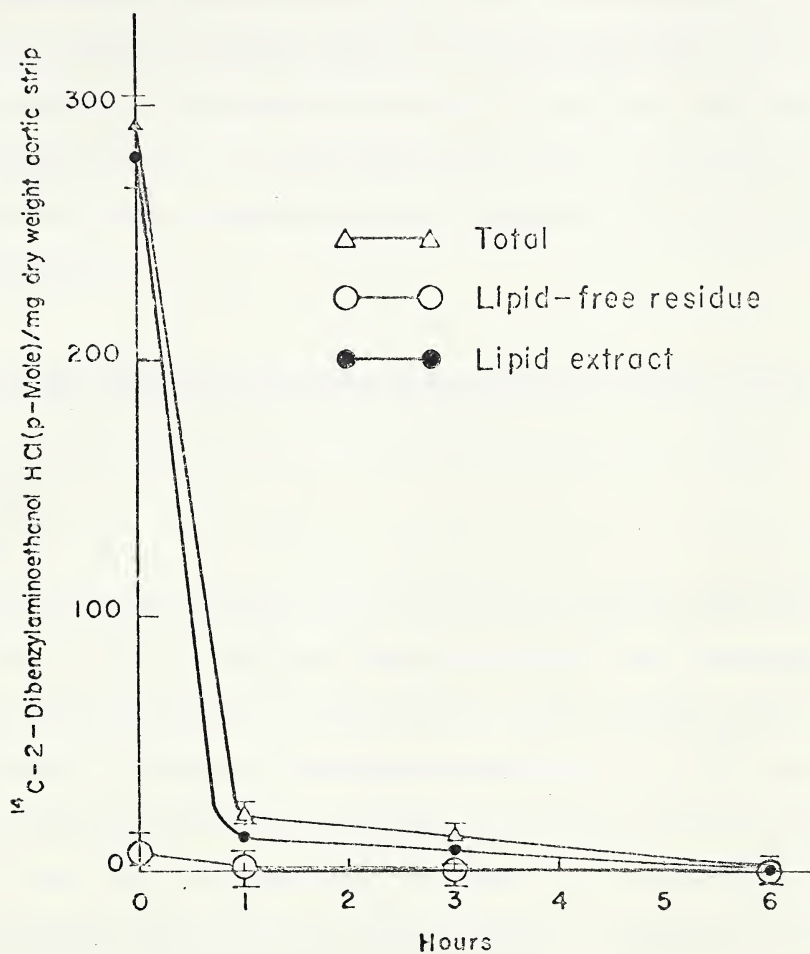


Fig. 20. Removal of Loosely Bound  $^{14}\text{C}$ -2-Dibenzylaminoethanol Hydrochloride from Rabbit Aortic Strips.



between lipid extracts and lipid-free residues after exposure of strips to  $^{14}\text{C}$ -dibenamine hydrochloride (Fig. 17) and  $^{14}\text{C}$ -2-dibenzylaminoethanol hydrochloride (Fig. 20) and washing for a 3 hr period. In the case of  $^{14}\text{C}$ -dibenamine hydrochloride labeled strips 80% of the total radioactivity was associated with non-lipid residue, while in the experiments with  $^{14}\text{C}$ -2-dibenzylaminoethanol hydrochloride virtually no radioactivity was associated with the non-lipid residue. It thus appears that it is the labeling of non-lipid material that is necessary to obtain effective blockade of  $\alpha$ -adrenergic receptors.

### C. Studies of the Binding of Dibenamine Hydrochloride to Rabbit Aortic Strips

#### Introduction

As pointed out in Chapter IIB, the amount of radioactivity observed in control strips in procedure I was surprisingly high and complicated the labeling approach proposed by Gaddum (1962). It was apparent that exposure of strips to unlabeled dibenamine hydrochloride ( $3 \times 10^{-6}$ ) for 20 min in the experiments described in Chapter IIB did not result in saturation of the tissue with the reagent and the tissue retained the capacity to take up  $^{14}\text{C}$ -dibenamine hydrochloride. It was therefore important to determine whether a method could be established to saturate the tissue with unlabeled dibenamine hydrochloride by either (1) increasing the time of exposure to this agent or (2) by increasing the concentration of this agent.



Experimental

i. The Uptake of  $^{14}\text{C}$ -Dibenamine Hydrochloride by Rabbit Aortic Strips after Exposure to This Agent for Various Time Periods

Eight rabbit aortic strips were exposed to  $^{14}\text{C}$ -dibenamine hydrochloride ( $3 \times 10^{-6}$ ) for the following time periods: strips 1 and 2, 10 min; strips 3 and 4, 30 min; strips 5 and 6, 60 min; and strips 7 and 8, 90 min. The strips were washed at 15 min intervals for 3 hr and the radioactivity associated with lipid extracts and lipid-free residues was determined. The results are shown in Fig. 21.

ii. The Uptake of  $^{14}\text{C}$ -Dibenamine Hydrochloride by Rabbit Aortic Strips after Exposure to Different Concentrations of This Agent

Eight rabbit aortic strips were exposed to the following concentrations of  $^{14}\text{C}$ -dibenamine hydrochloride for 20 min: strips 1 and 2,  $3 \times 10^{-8}$ ; strips 3 and 4,  $3 \times 10^{-7}$ ; strips 5 and 6,  $3 \times 10^{-5}$ ; and strips 7 and 8,  $3 \times 10^{-4}$ . The strips were washed for 3 hr at 15 min intervals and the radioactivity associated with lipid extracts and lipid-free residues of the strips was determined. The results are shown in Fig. 22.

iii. The Uptake of  $^{14}\text{C}$ -Dibenamine Hydrochloride by Rabbit Aortic Strips after Exposure to Different Concentrations of Unlabeled Dibenamine Hydrochloride

Eight rabbit aortic strips were exposed to the following concentrations of unlabeled dibenamine hydrochloride for 20 min: strips 1 and 2,  $3 \times 10^{-8}$ ; strips 3 and 4,  $3 \times 10^{-7}$ ; strips 5 and 6,  $3 \times 10^{-5}$ ; and strips



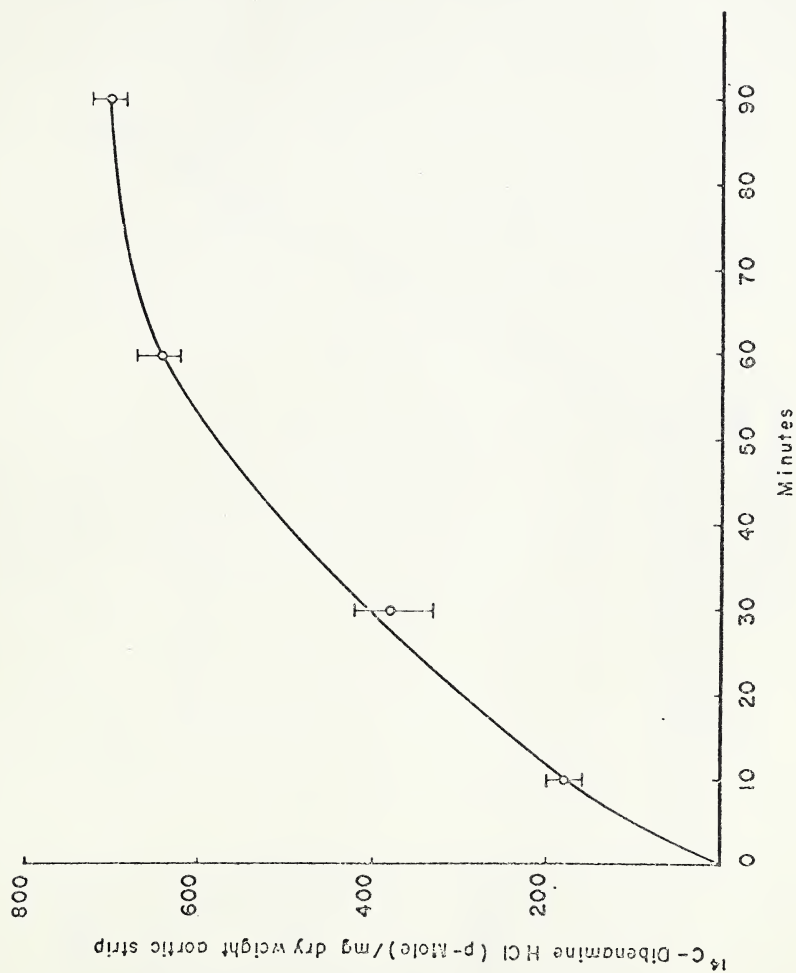


Fig. 21. The Uptake of  $^{14}\text{C}$ -Dibenamine Hydrochloride by Rabbit Aortic Strips after Exposure to This Agent ( $3 \times 10^{-6}$ ) for Various Time Periods.





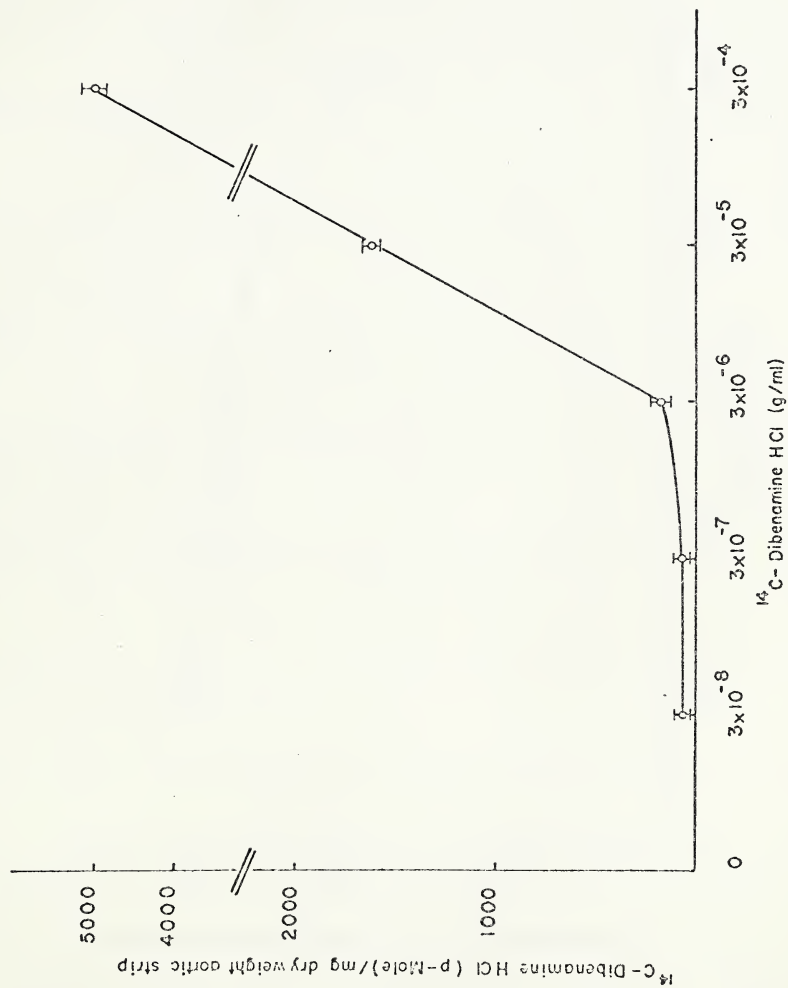


Fig. 22. The Uptake of  $^{14}\text{C}$ -Dibenamine Hydrochloride by Rabbit Aortic Strips after Exposure to Different Concentrations of This Agent.



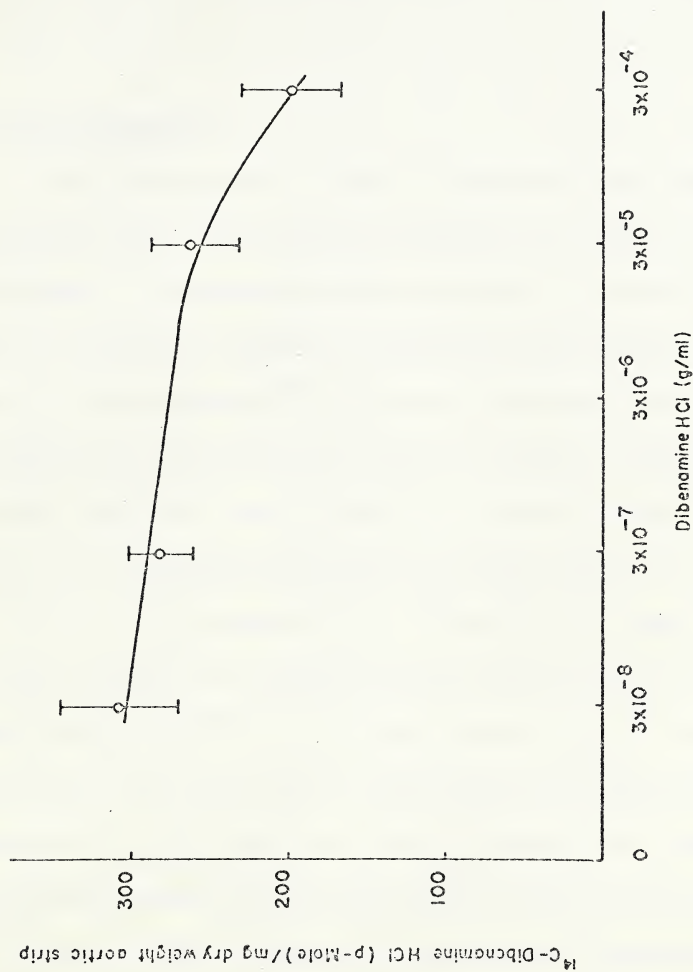


Fig. 23. The Uptake of  $^{14}\text{C}$ -Dibenamine Hydrochloride by Rabbit Aortic Strips after Exposure to Different Concentrations of Unlabeled Dibenamine Hydrochloride.



7 and 8,  $3 \times 10^{-4}$ . After one wash, strips were exposed to  $^{14}\text{C}$ -dibenamine hydrochloride ( $3 \times 10^{-6}$ ) for 20 min and washed at 15 min intervals for 3 hr. The radioactivity associated with lipid extracts and lipid-free residues of the strips was then determined. The results are shown in Fig. 23.

### Results and Discussion

The results shown in Fig. 21 indicated that rabbit aortic strips were saturated with  $^{14}\text{C}$ -dibenamine hydrochloride after a 90 min exposure to this agent. It therefore seemed desirable to extend the period of exposure of strips to unlabeled dibenamine hydrochloride from 20 to 90 min prior to labeling with  $^{14}\text{C}$ -dibenamine hydrochloride in order to reduce the radioactivity associated with control strips in procedure I. Before doing so an experiment was carried out to check that the tissue was indeed saturated with dibenamine hydrochloride after exposure to this agent ( $3 \times 10^{-6}$ ) for a 90 min period as indicated by the results of Fig. 21. Strips were exposed to a freshly prepared solution of  $^{14}\text{C}$ -dibenamine hydrochloride ( $3 \times 10^{-6}$ ) for the same period of time. The results (Table VII) show that the tissue was not saturated with dibenamine hydrochloride after exposure to this agent for a 90 min period. The probable reason for the apparent saturation as indicated by the flattening of the curve (Fig. 21) was the inactivation of the EI ion of dibenamine hydrochloride by interaction with water (Graham, 1962). Thus there does not appear to be an advantage in increasing the length of exposure of strips to unlabeled dibenamine hydrochloride in procedure I.

The studies summarized in Fig. 22 show that it is not possible to



TABLE VII. DISINTEGRATIONS PER MINUTE DETECTED IN RABBIT AORTIC STRIPS

Dis/min after (a) exposure to  $^{14}\text{C}$ -dibenzamine hydrochloride ( $3 \times 10^{-6}$  g/ml) for a 90-min period; (b) after re-exposure for a further 90-min period to a freshly prepared solution of this reagent ( $3 \times 10^{-6}$  g/ml).

Experiment No.	Total dis/min/mg Dry Weight of Strip	
	(a) First 90-min Exposure	(b) Second 90-min Exposure
1	746	1269
2	784	1421





saturate the aortic tissue with dibenamine hydrochloride by increasing the concentration of this agent. A sharp increase in uptake of firmly bound  $^{14}\text{C}$ -dibenamine hydrochloride occurs when the concentration is raised beyond  $3 \times 10^{-6}$ , which is the concentration required to completely block the  $\alpha$ -adrenergic receptors. It is of considerable interest to compare the shape of this uptake curve with the uptake curve of  $^3\text{H}$ -atropine obtained by Paton & Rang (1965) (Fig. 24). The latter workers measured the amount of  $^3\text{H}$ -atropine taken up by the longitudinal muscle of guinea-pig ileum after exposure to various concentrations of this agent. The uptake curve of  $^3\text{H}$ -atropine resembles the uptake curve of  $^{14}\text{C}$ -dibenamine hydrochloride in the following respects: (1) the amount of  $^3\text{H}$ -atropine and  $^{14}\text{C}$ -dibenamine hydrochloride taken up by the tissue after exposure to concentrations ranging from  $1 \times 10^{-9}$  to  $1 \times 10^{-7}$  M of these agents was very small; (2) uptake of  $^3\text{H}$ -atropine and  $^{14}\text{C}$ -dibenamine hydrochloride increases very rapidly as the concentration exceeds a certain critical value.

In order to explain the nature of the  $^3\text{H}$ -atropine uptake curve Paton & Rang attempted to fit a theoretical curve derived from a modified Langmuir adsorption equation to their experimental curve. By means of this type of analysis, they were able to distinguish three components of atropine uptake in the tissue as follows: (1) a binding site with a capacity of 180 p-moles atropine/g tissue and an equilibrium constant of  $1.1 \times 10^{-9}$  M, which they believed to be related to the acetylcholine receptor; (2) a binding site of capacity about 1,000 p-moles atropine/g tissue and an equilibrium constant of  $5 \times 10^{-7}$  M, which they believed to be related to the non-specific sites of atropine uptake; (3) a non-saturable compartment in which uptake of atropine into the intracellular fluid space is in direct proportion to the



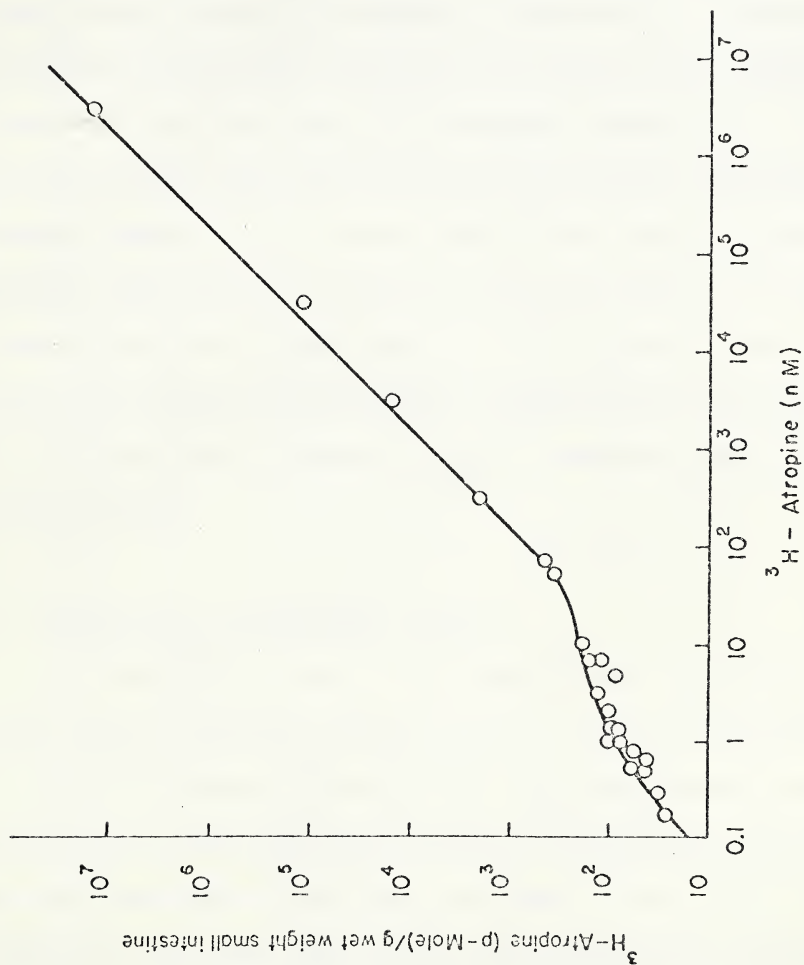


Fig. 24. The Uptake of  $^3\text{H}$ -Atropine by the Longitudinal Muscle of Guinea-Pig Ileum (Paton & Rang, 1965).



external concentration of this agent. It should be pointed out that the  $^3\text{H}$ -atropine uptake represents the amount of  $^3\text{H}$ -atropine retained in the tissue after 4 hr exposure to different concentrations of this agent. On the other hand the amount of  $^{14}\text{C}$ -dibenamine hydrochloride taken up by the tissue in our studies is a measure of that portion of firmly bound  $^{14}\text{C}$ -dibenamine hydrochloride retained by the tissue after exposure to different concentrations of this agent for 20 min and washing for a 3 hr period. For the above reason the uptake curve of  $^{14}\text{C}$ -dibenamine hydrochloride cannot be directly compared to the uptake curve of  $^3\text{H}$ -atropine. It is, however, probable that the first portion of the curve (Fig. 22;  $^{14}\text{C}$ -dibenamine hydrochloride concentration less than  $3 \times 10^{-6}$ ) represents uptake of  $^{14}\text{C}$ -dibenamine hydrochloride by  $\alpha$ -adrenergic receptors while the second portion of the curve represents combination of  $^{14}\text{C}$ -dibenamine hydrochloride with non-specific sites.

#### D. Repetition of Procedures I and II

Procedures I and II were repeated with the modification that rabbit aortic strips were washed for 3 hr at 15 min intervals after labeling with  $^{14}\text{C}$ -dibenamine hydrochloride. The radioactivity present in the lipid extracts and lipid-free residues of experimental and control strips was then determined. The results are shown in Table VIII. Several differences were noted when these results were compared with those obtained in Chapter II: (1) The amount of  $^{14}\text{C}$ -dibenamine hydrochloride taken up is greater when tissues are mounted as single strips rather than as loops. Apparently there was inadequate access of  $^{14}\text{C}$ -dibenamine hydrochloride in our experimental arrangement described in Chapter II. (2) The percentage



TABLE VIII. THE DISINTEGRATIONS PER MINUTE DETECTED IN THE LIPID EXTRACTS AND LIPID-FREE RESIDUES OF EXPERIMENTAL AND CONTROL RABBIT AORTIC STRIPS.

The total dis/min/mg dry weight of strip is the sum of the dis/min/mg of the lipid extract and lipid-free residue.

Aortic Strip	Dis/min in Lipid-Free residue/mg dry weight of strip $\pm$ S.D.		Dis/min in Lipid Extract /mg dry weight of strip $\pm$ S.D.		Total dis/min/mg Dry weight of strips $\pm$ S.D.	
	Labeling Procedure I	Labeling Procedure II	Labeling Procedure I	Labeling Procedure II	Labeling Procedure I	Labeling Procedure II
Control	186 $\pm$ 28 (10)*	302 $\pm$ 38 (8)	43 $\pm$ 6 (10)	58 $\pm$ 15 (8)	229 $\pm$ 33 (10)	360 $\pm$ 50 (8)
Experimental	204 $\pm$ 39 (10)	211 $\pm$ 38 (8)	52 $\pm$ 9 (10)	38 $\pm$ 10 (8)	256 $\pm$ 46 (10)	249 $\pm$ 47 (8)
P Value	> 0.05	< 0.05	< 0.05	< 0.05	> 0.05	< 0.05

\* Numbers enclosed by parentheses indicate the number of experiments performed.





TABLE IX. THE DISINTEGRATIONS PER MINUTE DETECTED IN THE COMPONENTS OF LIPID EXTRACTS OF EXPERIMENTAL AND CONTROL RABBIT AORTIC STRIPS LABELED BY PROCEDURE II, WITH MODIFICATION.

R <sub>f</sub> Value	10 µg <sup>14</sup> C-dibenamine HCl	Control Strip	Experimental Strip
	Total dis/min per segment of chromatogram	Total dis/min per segment of chromatogram/ 100 mg dry weight of aortic strip	
0-0.04	0	40	80
0.04-0.13*	4	30	50
0.13-0.29†	33	60	90
0.29-0.50‡	8	400	400
0.50-0.70	20	200	200
0.70-1.00	13,688	1,500	1,000
Total	13,753	2,230	1,820

\* Range for sphingomyelin.

† Range for phosphatidyl serine.

‡ Range for cephalin.



radioactivity detected in the lipid fraction in procedure I was the same as that detected in the lipid fraction in procedure II (15-20%). This was not the case in our study described in Chapter II. A study of the distribution of radioactivity in the lipid components of the lipid extracts (Table IX) confirmed those obtained in Chapter II.

The results obtained in the present study indicate that procedure II is the procedure of choice. It was therefore planned to use procedure II in our further studies. It is necessary to point out at this stage of investigation, however, that even with procedure II, complications arise in the interpretation of our results. The major complication is the fact that epinephrine undoubtedly masks sites of dibenamine hydrochloride uptake other than the  $\alpha$ -adrenergic receptors. In particular, epinephrine would mask sites in the nerve terminals with which dibenamine hydrochloride can combine (Furchgott & Kirpekar, 1963; Furchgott et al., 1963). In Chapter V experiments are described in which this complication is avoided.

#### E. The Use of Phentolamine as a Protecting Agent in Labeling Studies

##### Introduction

In the labeling studies described in Chapters II and IIID a high concentration of epinephrine ( $1 \times 10^{-4}$ ) was used to protect the  $\alpha$ -adrenergic receptors from combination with  $^{14}\text{C}$ -dibenamine hydrochloride. Waud (1962) has pointed out that the use of a high concentration of an agent such as epinephrine results in the protection of non-specific sites as well as  $\alpha$ -adrenergic receptor sites from combination with dibenamine hydrochloride. Since the reversible  $\alpha$ -adrenergic receptor blocking agent, phentolamine



(Fig. 25) protects the receptors from combination with dibenamine hydrochloride when present in a much lower concentration than epinephrine (Furchgott, 1966), it was proposed to use phentolamine as a protecting agent instead of epinephrine. A series of experiments was performed to establish the concentration of phentolamine necessary to protect the  $\alpha$ -adrenergic receptors from blockade by  $^{14}\text{C}$ -dibenamine hydrochloride ( $3 \times 10^{-6}$ ). We then repeated procedure II using phentolamine as a protecting agent.

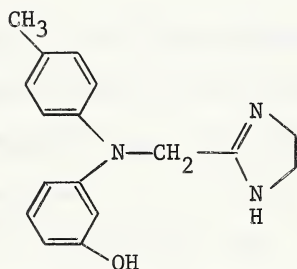


Fig. 25. Chemical Structure of Phentolamine

### Experimental

#### 1. Inhibition of $^{14}\text{C}$ -Dibenamine Hydrochloride Uptake by Phentolamine

Rabbit aortic strips were prepared and mounted as single strips in organ baths as indicated in Fig. 9B. The responses of the aortic strips to several concentrations of epinephrine were recorded. The strips were exposed to phentolamine methanesulfonate ( $3 \times 10^{-7}$ ) for 5 min and  $^{14}\text{C}$ -dibenamine hydrochloride ( $3 \times 10^{-6}$ ) added. After a further 20 min period the strips were washed for 3 hr at 15 min intervals with Krebs's bicarbonate solution. The responses of strips to various concentrations of epinephrine



were then redetermined. As a control the above experimental procedure was repeated with the omission of phentolamine methanesulfonate.

The above experiment was repeated several times keeping all the factors constant but varying the phentolamine concentration. Thus in the second experiment phentolamine methanesulfonate ( $3 \times 10^{-6}$ ) was used, and in a third experiment phentolamine methanesulfonate ( $1 \times 10^{-5}$ ) was used. The determination of the degree of protection and of the radioactivity was performed as described in Chapter II. The results are shown in Table X.

ii. Phentolamine ( $1 \times 10^{-5}$ ) as a Protecting Agent in Procedure II

The responses of rabbit aortic strips to several concentrations of epinephrine were recorded (Fig. 26A). The strips were treated with phentolamine methanesulfonate ( $1 \times 10^{-5}$ ) for 5 min and  $^{14}\text{C}$ -dibenamine hydrochloride ( $3 \times 10^{-6}$ ) added (Fig. 26B). After a further 20 min period the strips were washed for 3 hr at 15 min intervals in Krebs's bicarbonate solution and the responses to various concentrations of epinephrine redetermined (Fig. 26C). As controls, the experiments were repeated with the omission of the protecting dose of phentolamine methanesulfonate ( $1 \times 10^{-5}$ ) in the first step. The radioactivity associated with experimental and control strips is shown in Table XI.

Results and Discussion

Furchgott (1966) observed that phentolamine ( $1.15 \times 10^{-5}$ ) protected the  $\alpha$ -adrenergic receptors of rabbit aortic strips from combination with dibenamine hydrochloride ( $1 \times 10^{-6}$ ). The results reported in Table X show that phentolamine methanesulfonate at a concentration of  $3 \times 10^{-6}$  or  $1 \times 10^{-5}$  afforded complete protection against blockade by dibenamine





TABLE X. THE INHIBITION OF  $^{14}$ C-DIBENAMINE HYDROCHLORIDE UPTAKE IN RABBIT  
AORTIC STRIPS BY PHENTOLAMINE METHANESULFONATE

Concentration of Phentolamine Methanesulfonate (g/ml)	Remaining Sensitivity to Epinephrine (% of original)	Dis/min/mg dry weight of strip $\pm$ S.D.	
		Lipid Extract	Lipid-Free Residue
0	--	64 $\pm$ 18 (10)**	333 $\pm$ 93 (10)
3 x 10 <sup>-7</sup>	< 8%	54 $\pm$ 12 (5)	277 $\pm$ 49 (5)
3 x 10 <sup>-6</sup>	100%	67 $\pm$ 22 (5)	323 $\pm$ 92 (5)
1 x 10 <sup>-5</sup>	100%	44 $\pm$ 6* (4)	229 $\pm$ 15* (4)

\* Significantly different from control at 0.05 level.

\*\* Numbers enclosed by parentheses indicate the number of experiments performed.



2 minutes

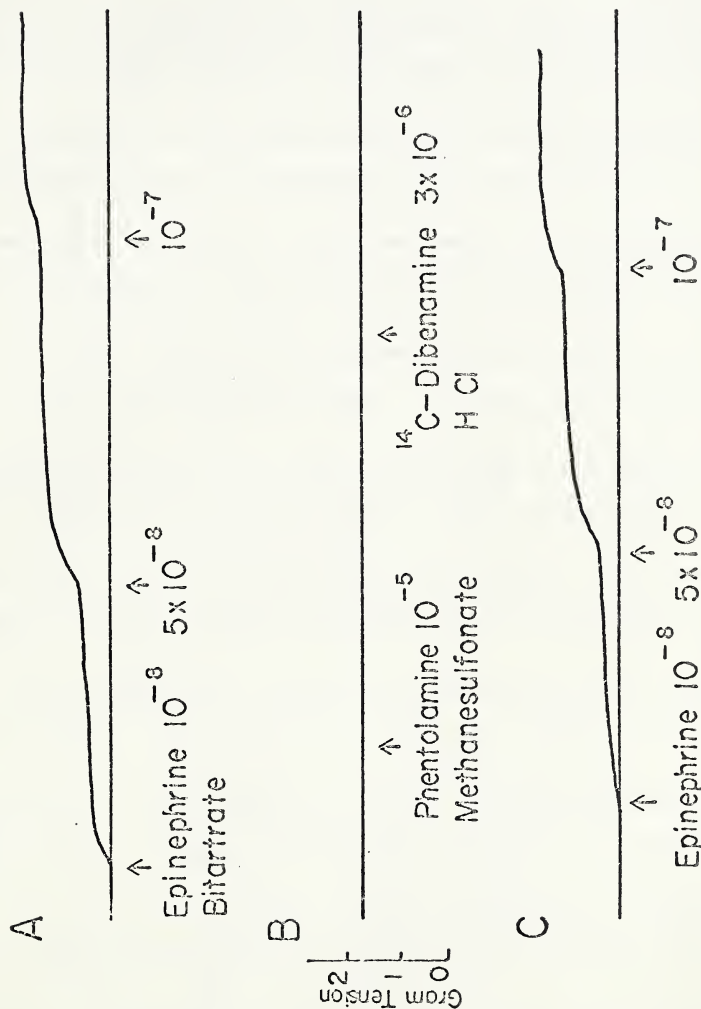


Fig. 26. The Response of Rabbit Aortic Strips to Epinephrine in Labeling Studies Using Phentolamine as Protecting Agent.



hydrochloride ( $3 \times 10^{-6}$ ) in this tissue. However, using phentolamine methanesulfonate at a concentration of  $3 \times 10^{-7}$ , the degree of protection dropped to less than 10%. It was surprising that despite complete receptor protection with phentolamine ( $3 \times 10^{-6}$ ) and ( $1 \times 10^{-5}$ ) only with the latter concentration was a significant difference in radioactivity between control and protected strips observed. Thus there is no apparent correlation between the degree of protection afforded by phentolamine and the difference in radioactivity between experimental and control strips. It will be recalled that Clark's receptor theory was based on the assumption that the intensity of response is directly related to the number of receptors occupied by an active drug. However, this assumption was shown to be incorrect by Furchgott (1955) and Nickerson (1956) who showed that maximum drug effects could be observed when only a small percentage of available receptors was occupied. Thus it is possible that the lower concentration of phentolamine methanesulfonate ( $3 \times 10^{-6}$ ) protected only a small fraction of the total  $\alpha$ -adrenergic receptors from combination with  $^{14}\text{C}$ -dibenamine hydrochloride. For this reason significant differences in radioactivity between experimental and control strips were not observed. However, the small number of receptors protected from combination with  $^{14}\text{C}$ -dibenamine hydrochloride sufficed to give a maximum response when occupied by epinephrine.

We have used a concentration of phentolamine methanesulfonate ( $1 \times 10^{-5}$ ) which consistently produced complete receptor protection and inhibition of  $^{14}\text{C}$ -dibenamine hydrochloride uptake (Table XI). A significant increment in radioactivity was noted in both lipid extracts and lipid-free residues of the control strips as compared to the corresponding fractions of experimental strips. This finding corresponded to our previous



TABLE XI. THE DISINTEGRATIONS PER MINUTE DETECTED IN THE LIPID EXTRACTS AND LIPID-FREE RESIDUES OF CONTROL AND EXPERIMENTAL RABBIT AORTIC STRIPS USING PHENTOLAMINE AS PROTECTING AGENT.

Aortic Strip	Labeling Conditions	Dis/min/mg of Dry Weight of Strip		
		<u>Lipid-Free Residue</u>	<u>Lipid Extract</u>	<u>Total</u>
Control	$^{14}\text{C}$ Dibenamine HCl 3 x 10 <sup>-6</sup> g/ml	373 ± 54 (9)*	68 ± 10 (9)	441 ± 59 (9)
Experimental	Phentolamine methanesulfonate 1 x 10 <sup>-5</sup> g/ml	266 ± 74 (10)	48 ± 12 (10)	314 ± 86 (10)
	$^{14}\text{C}$ Dibenamine HCl 3 x 10 <sup>-6</sup> g/ml			
P Value		< 0.05	< 0.05	< 0.05

The total dis/min/mg dry weight of strip is the sum of the dis/min/mg of the lipid extract and lipid-free residue.

\* Numbers enclosed by parentheses indicate the number of experiments performed.





observations using epinephrine as a protecting agent.

As pointed out in the introduction, phentolamine affords receptor protection at a lower concentration than epinephrine and thus the problem of protection of non-specific sites is reduced. However, complications remain in the interpretation of our results. Iversen (1965) has shown that phentolamine inhibits the uptake of norepinephrine into the storage sites of adrenergic nerve terminals of rat heart. For this reason it is likely that phentolamine prevents the combination of  $^{14}\text{C}$ -dibenzamine hydrochloride with the  $\alpha$ -adrenergic receptors as well as norepinephrine uptake sites. The difference in radioactivity between experimental and control strips is thus not a measure of the  $\alpha$ -adrenergic receptor sites alone.

#### F. Relationship Between Uptake of $^{14}\text{C}$ -Dibenzamine Hydrochloride and Blockade of $\alpha$ -Adrenergic Receptors

##### Introduction

To explore the relationship between the amount of  $^{14}\text{C}$ -dibenzamine hydrochloride taken up by aortic strips and the degree of blockade, strips were exposed to concentrations of this agent ranging from  $3 \times 10^{-8}$  to  $3 \times 10^{-6}$  for various time periods.

##### Experimental

(a) The responses of 12 rabbit aortic strips to several concentrations of epinephrine was recorded. From the average of these responses a cumulative dose-response curve was constructed (Fig. 27a). Aortic strips were then exposed to the following concentration of  $^{14}\text{C}$ -dibenzamine



hydrochloride for 20 min: strips 1, 2 and 3,  $3 \times 10^{-8}$ ; strips 4, 5 and 6,  $6 \times 10^{-8}$ ; strips 7, 8 and 9,  $9 \times 10^{-8}$ ; and strips 10, 11 and 12,  $1.2 \times 10^{-7}$ . The strips were washed for 3 hr at 15 min intervals and cumulative epinephrine dose-response curves were re-determined (Fig. 27b,c,d,e). The amount of radioactivity associated with lipid extracts and lipid-free residues of the strips was determined (Table XII).

(b) The responses of 12 rabbit aortic strips to various concentrations of epinephrine were recorded. From the average of these responses a cumulative dose-response curve was constructed (Fig. 28a). Six of the above aortic strips were then exposed to  $^{14}\text{C}$ -dibenzamine hydrochloride ( $1.2 \times 10^{-7}$ ) for the following time periods: strips 1 and 2, 2 min; strips 3 and 4, 4 min; and strips 5 and 6, 6 min. The strips were washed for 3 hr at 15 min intervals and cumulative epinephrine dose-response curves were again determined (Fig. 28b,c,d). The radioactivity associated with lipid extracts and lipid-free residues of the strips were determined (Table XII).

(c) The remaining six aortic strips were exposed to  $^{14}\text{C}$ -dibenzamine hydrochloride ( $3 \times 10^{-6}$ ) for the following time periods: strips 1 and 2, 2 min; strips 3 and 4, 4 min; and strips 5 and 6, 6 min. The strips were washed for 3 hr at 15 min intervals and cumulative epinephrine dose-response curves were re-determined (Fig. 28e,f). The radioactivity associated with lipid extracts and lipid-free residues of the strips was determined (Table XII).

## Results and Discussion

The data presented in Table XII and Figs. 27 and 28 were utilized



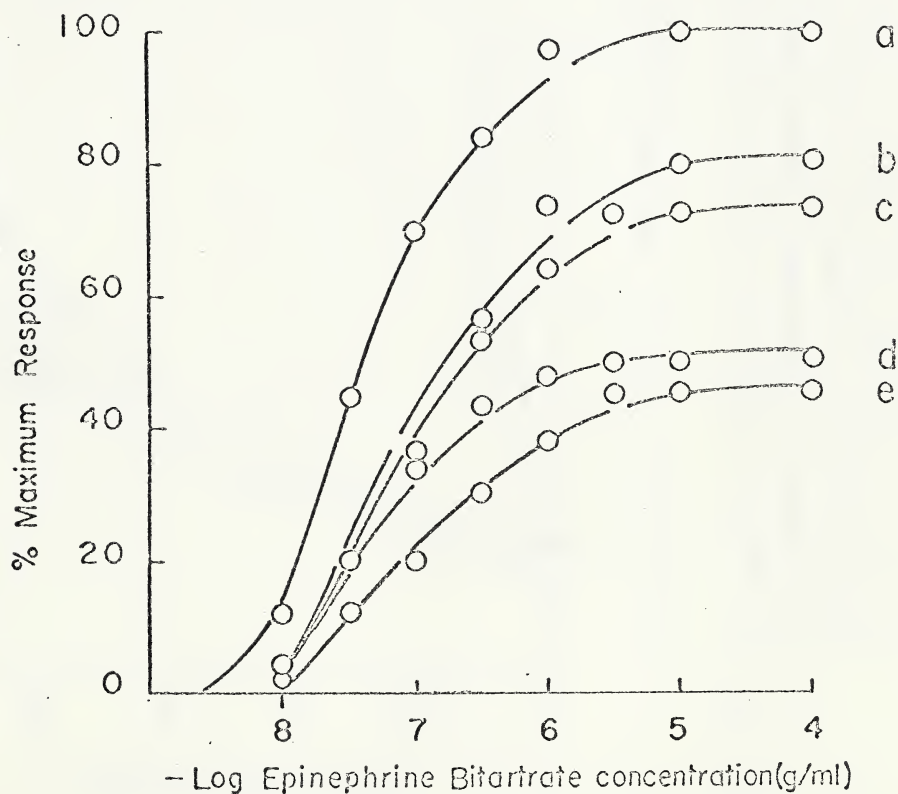


Fig. 27. The Effect of  $^{14}\text{C}$ -Dibenamine Hydrochloride on the Response of Rabbit Aortic Strips to Epinephrine. a, Control; b,  $3 \times 10^{-8}$ ; c,  $6 \times 10^{-8}$ ; d,  $9 \times 10^{-8}$ ; e,  $1.2 \times 10^{-7}$ .



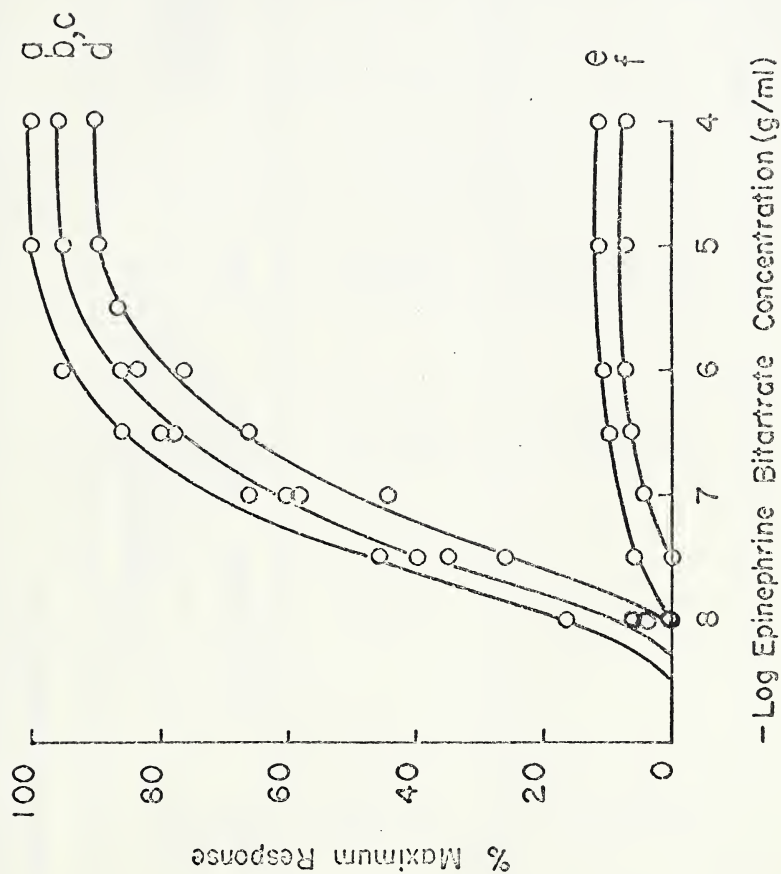


Fig. 28. The Response of Rabbit Aortic Strips to Epinephrine after Exposure to  $^{14}\text{C}$ -Dibenzamine Hydrochloride for Various Time Periods. a, Control; b,  $1.2 \times 10^{-7}$  for 2 min; c,  $1.2 \times 10^{-7}$  for 4 min; d,  $1.2 \times 10^{-7}$  for 6 min; e,  $3 \times 10^{-6}$  for 2 min; f,  $3 \times 10^{-6}$  for 4 min.





TABLE XII. RELATIONSHIPS BETWEEN THE UPTAKE OF  $^{14}\text{C}$ -DIBENAMINE HYDROCHLORIDE AND THE BLOCKADE OF  $\alpha$ -ADRENERGIC RECEPTORS.

Concentration of $^{14}\text{C}$ -Dibenamine HCl (g/ml)	Exposure Time (min)	$^{14}\text{C}$ -Dibenamine HCl (p-mole)/mg Dry Weight of Strip			% Maximum Response to Epinephrine
		Lipid Extract	Lipid-Free Residue	Total	
$3 \times 10^{-8}$	20	0	2.8	2.8	80
$6 \times 10^{-8}$	20	0	3.8	3.8	75
$9 \times 10^{-8}$	20	0	8.1	8.1	50
$1.2 \times 10^{-7}$	2	0	1.8	1.8	95
	4	0	1.8	1.8	90
	6	0	2.8	2.8	80
	20	0.9	9.9	10.8	45
$3 \times 10^{-6}$	2	4.7	29.2	34.0	12
	4	10.4	52.8	63.2	8
	6	15.1	63.2	78.3	0



to construct the curve (Fig. 29) which shows the relationship between the amount of  $^{14}\text{C}$ -dibenamine hydrochloride and the maximum epinephrine responses attainable. It was of interest that a considerable degree of irreversible  $\alpha$ -adrenergic blockade (approximately 50%) could be achieved with the  $^{14}\text{C}$ -dibenamine hydrochloride located exclusively in the lipid-free residue. This observation shows that it is unlikely that a lipid component is involved in the  $\alpha$ -adrenergic receptor site. For this reason we abandoned the idea (Chapter IID) of carrying out a more extensive study of the radioactivity associated with the lipid fraction. The data obtained in this experiment have been used to obtain an estimate of the number of  $\alpha$ -adrenergic receptors in rabbit aorta. This will be discussed in Chapter VI.



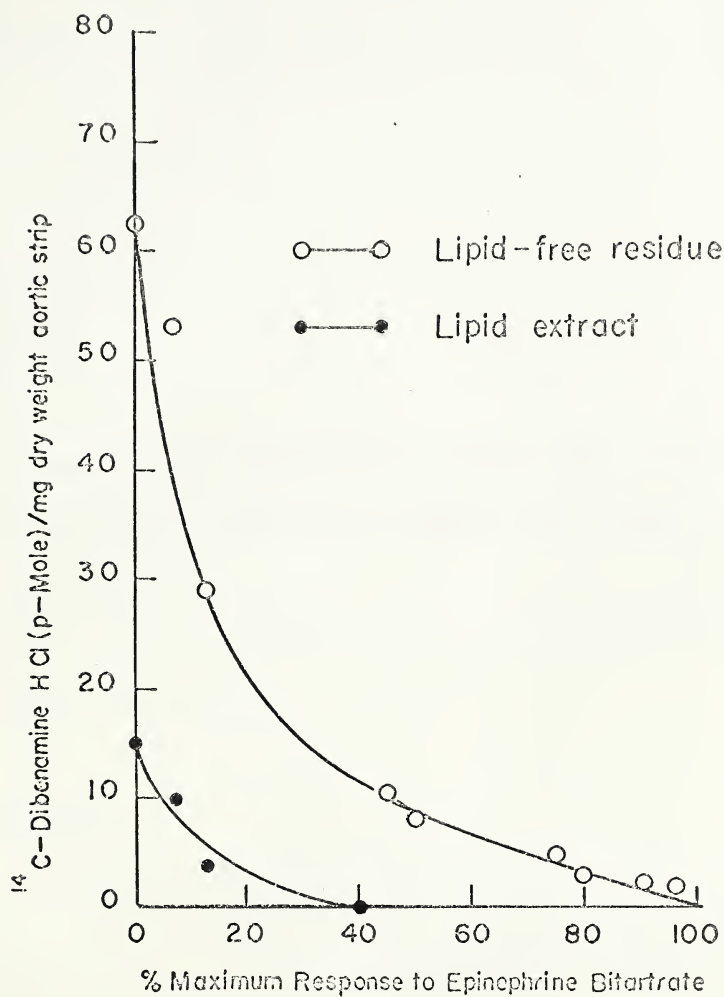


Fig. 29. The Relationship Between the Uptake of  $^{14}\text{C}$ -Dibenamine Hydrochloride and the Blockade of  $\alpha$ -Adrenergic Receptors in Rabbit Aortic Strips.



CHAPTER IV    DISTRIBUTION OF RADIOACTIVITY IN SUBCELLULAR  
FRACTIONS OF LABELED RABBIT AORTA





## Introduction

The next step in our study was to attempt the isolation of a  $^{14}\text{C}$ -dibenamine-receptor complex. Takagi, Akao & Takahashi (1965) labeled the acetylcholine receptors of intestinal smooth muscle with  $^3\text{H}$ -labeled dibenamine using atropine as a receptor protecting agent. They reported that subcellular fractionation of the labeled tissue was useful as a preliminary procedure for the isolation of the  $^3\text{H}$ -dibenamine-receptor complex. For this reason we have undertaken the separation of  $^{14}\text{C}$ -dibenamine hydrochloride labeled rabbit aortic tissue into its subcellular components. Rabbit aortic strips were labeled with  $^{14}\text{C}$ -dibenamine hydrochloride by procedure II using epinephrine as protecting agent. Control and experimental strips were homogenized and separated into subcellular components by differential centrifugation according to the procedure of Whereat (1966). The protein content and the radioactivity associated with subcellular fractions was determined.

## Experimental

### i. Preparation of Subcellular Fractions of Rabbit Aorta

The procedure of Whereat (1966) was used for the isolation of the subcellular components of rabbit aorta at  $4^\circ$ . A fine mince was prepared from rabbit aortic strips with the aid of a pair of small scissors. The minced tissue was placed in the upper bowl of an all glass TenBroeck tissue grinder (Kontes Glass Co.) and 30 ml of 0.28 M sucrose solution (containing  $5 \times 10^{-5}$  M EDTA) per gram of minced tissue was added to the barrel. The pestle was slowly rotated by hand while small amounts of mince



were allowed to fall from the bowl into the barrel. The homogenates were fractionated according to the scheme shown in Fig. 30 and described in detail below. The homogenate (H) was centrifuged at  $800 \times g$  for 6 min and the supernatant transferred to a second tube. The residue, containing nuclei (N) was resuspended in fresh 0.28 M sucrose solution and centrifuged at  $800 \times g$  for 6 min. The combined supernatant (MES) (see Fig. 30) was centrifuged at  $10,000 \times g$  for 15 min in a Beckman Model L 4 ultracentrifuge. The residue, containing mitochondria (M) was resuspended in fresh 0.28 M sucrose solution and recentrifuged at  $10,000 \times g$  for 15 min. The combined supernatant (ES) was centrifuged at  $100,000 \times g$  for 90 min. The supernatant (S) was removed leaving a residue containing microsomes (E).

ii. Determination of the Protein Content and Radioactivity of Each Subcellular Fraction

- (a) Method of Lowry as modified by Miller (1959) for protein determination

Reagents:

- (1) Dilute Folin-phenol reagent was prepared by diluting 5 ml of Folin-phenol reagent (Fisher Sci. Co.) with 50 ml of demineralized distilled water.
- (2) 1% Copper sulfate solution
- (3) 2% Sodium potassium tartrate solution
- (4) 10% Sodium carbonate in 0.5 M sodium hydroxide solution
- (5) Copper reagent was freshly prepared by adding 1 ml of (2) and 1 ml of (3) into 20 ml of (4).

A stock solution of protein was prepared by dissolving 100 mg of



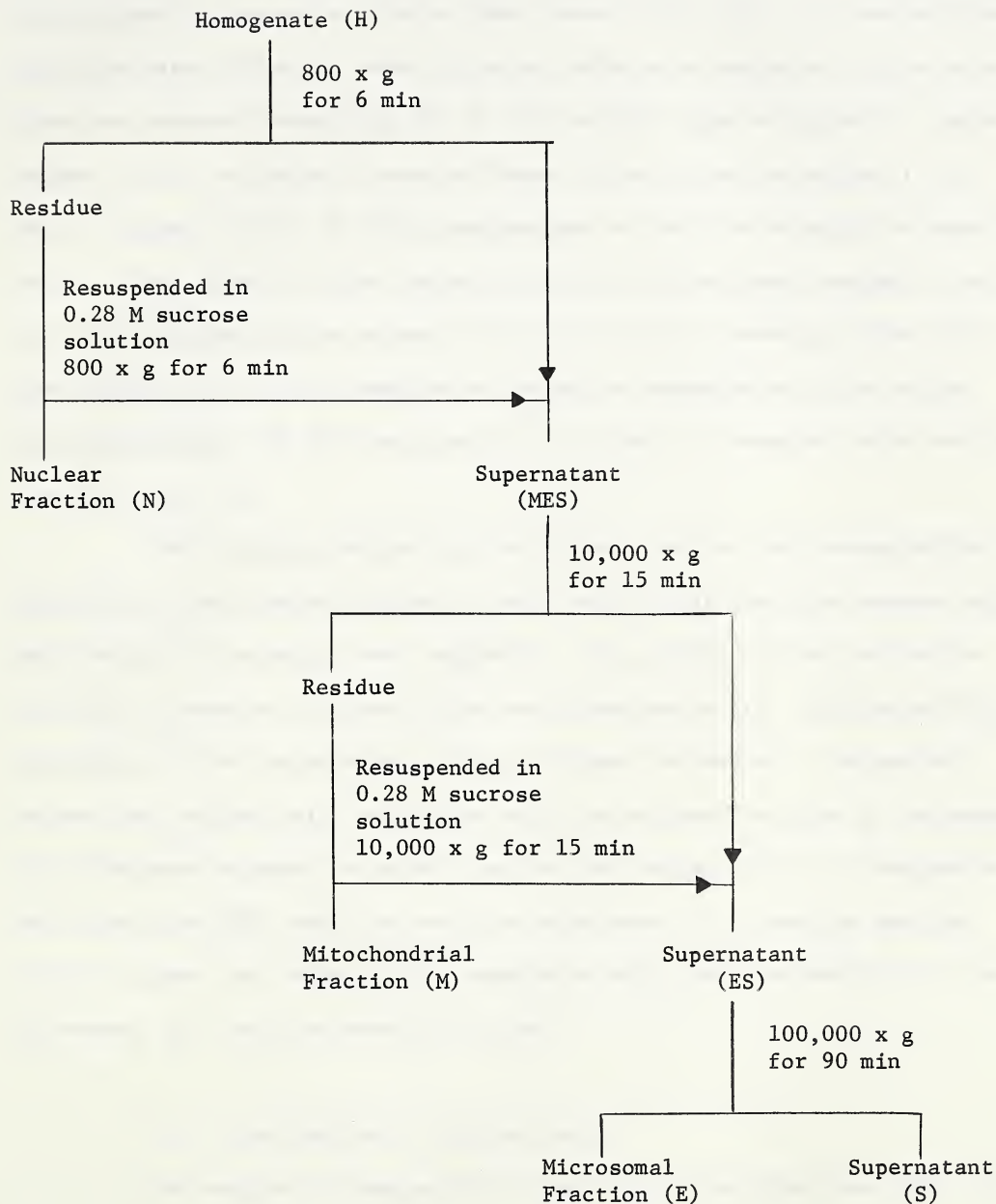


Fig. 30. Scheme for Preparation of Subcellular Fractions of Rabbit Aorta.



crystallized bovine serum albumin (Mann Research Lab. Inc.) in demineralized distilled water (50 ml). From this stock solution a series of dilute solutions was prepared containing 20, 65, 100 and 200  $\mu\text{g/ml}$  of protein. Copper reagent (1 ml) was added to each of these dilute protein solutions (1 ml) and to aliquots (1 ml) of dilute suspensions of cellular material as described below. After 10 min at room temperature dilute Folin-phenol reagent (3 ml) was added and the mixture warmed at  $50^\circ$  for 10 min to develop a blue color. Upon cooling, the optical density at 540  $\text{m}\mu$  was measured with the Unicam spectrophotometer (SP 600) using water as a blank. A standard curve was constructed (Fig. 31).

The following indirect procedure was used for the determination of radioactivity and protein content of each subcellular fraction because only small amounts of material were available: the protein content and radioactivity of measured aliquots of the whole homogenate (H), supernatant MES, supernatant ES and supernatant S (Fig. 30) were determined. The protein content and radioactivity associated with the nuclear fraction (N) represent the difference between the protein content and radioactivity of homogenate (H) and supernatant MES, and thus could be calculated. In a similar way the protein content and radioactivity associated with the mitochondrial (M) and microsomal (E) fractions were calculated.

#### (b) Determination of radioactivity

The samples of homogenate (H) and supernatant fractions, MES, ES and S, were prepared for radioactivity determination as follows: the samples (0.1 ml) were digested at  $70^\circ$  in 0.1 ml of 5N KOH solution. For use as blank 0.1 ml of the original 0.28 M sucrose solution was digested with 0.1 ml of







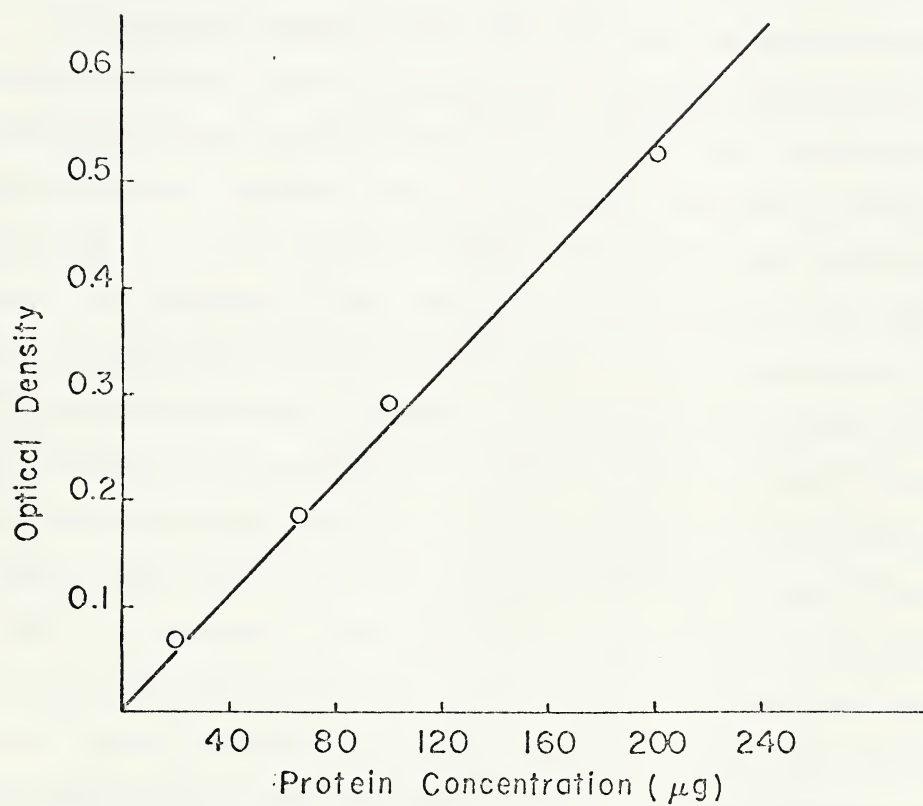


Fig. 31. Standard Curve for Protein Determination.



5N KOH solution. Reagent grade methanol (5.3 ml) and scintillation solution (12.5 ml) prepared as described in Chapter II were added to the cooled digests. The radioactivity of the samples and the blank was determined.

### Results and Discussion

The results recorded in Table XIII show that the radioactivity is distributed between all subcellular components. Moreover, significant differences exist between the radioactivity in all subcellular components of control strips when compared to the same subcellular components of experimental strips. It is of interest to compare these results with those reported by Takagi, Akao & Takahashi (1965) (Table XIV) in an analogous study using dog intestinal smooth muscle labeled by procedure I. In the final column of Table XIV they have recorded the differences in radioactivity between subcellular components of experimental and control tissues as  $E - C/C \%$ , where  $E$  = radioactivity in experimental and  $C$  = radioactivity in control components. In comparing these results with those recorded in the final column of our Table XIII it is necessary to point out the following facts: (1) the results in our final column are recorded as  $C - E/E \%$  since we have used labeling procedure II which is the reverse of their labeling procedure; (2) unlike our microsomal fraction which represents material sedimenting between 10,000 and 100,000  $\times g$ , they have subdivided this fraction into material sedimenting between 10,000 and 50,000  $\times g$  ( $P_2$ ) and material sedimenting between 50,000 and 105,000  $\times g$  ( $P_3$ ). Despite the differences in our two procedures it was nevertheless of interest that the largest difference in radioactivity was found in the microsomal fraction and the second largest difference in the mitochondrial fraction. In our procedure these differences in radioactivity



TABLE XIII. THE DISTRIBUTION OF RADIOACTIVITY IN SUBCELLULAR FRACTIONS OF CONTROL AND EXPERIMENTAL RABBIT AORTIC STRIPS.

Fraction	Protein Content ± S.D. (mg)		Total dis/min ± S.D.		Dis/min/mg Protein ± S.D.		$\frac{C - E}{E} \%$
	C	E	C	E	C	E	
Homogenate	7.8 ± 1.7 (6)	8.2 ± 1.4 (6)	5511 ± 1404 (6)	3977 ± 1238 (6)	696 ± 69 (6)	486* ± 85 (6)	43.2
Nuclear	4.2 ± 1.1 (6)	5.1 ± 1.5 (6)	2706 ± 792 (6)	2247 ± 631 (6)	618 ± 149 (6)	486* ± 85 (6)	27.2
Mitochondrial	2.6 ± 0.7 (6)	2.4 ± 0.5 (6)	1917 ± 164 (6)	1043 ± 422 (6)	722 ± 195 (6)	475* ± 85 (6)	52.0
Microsomal	0.5 ± 0.1 (6)	0.4 ± 0.1 (6)	490 ± 171 (6)	209 ± 82 (6)	1072 ± 274 (6)	582* ± 182 (6)	84.2
Supernatant	0.4 ± 0.08 (6)	0.5 ± 0.01 (6)	328 ± 152 (6)	330 ± 174 (6)	781 ± 242 (6)	721 ± 277 (6)	8.3

C = Control; E = Experimental

\* Significant difference at 0.05 level.



TABLE XIV. THE DISTRIBUTION OF RADIOACTIVITY IN SUBCELLULAR FRACTIONS OF CONTROL AND EXPERIMENTAL DOG INTESTINAL SMOOTH MUSCLE REPORTED BY TAKAGI, AKAO AND TAKAHASHI (1965).

Fraction	Total Nitrogen (mg/g of tissue)	Dis/min/0.1 mg of Nitrogen			$\frac{E - C}{C} \%$
		E	C	E-C	
H	11.01	18100	13500	4600	34.0
P <sub>1</sub>	6.84	15407	13659	1748	12.8
P <sub>2</sub>	0.43	22900	17100	5800	33.9
P <sub>3</sub>	0.49	48547	33200	15347	46.3
P <sub>4</sub>	0.56	40620	36500	4120	11.3
S	1.46	10735	9545	1190	12.5
P <sub>5</sub>	0.63	29650	20300	9350	46.0

C = Control; E = Experimental.

H = Homogenate, P<sub>1</sub> = Material sedimented at 1,000 x g.

P<sub>2</sub> = Material sedimented at 10,000 x g. S = Supernatant.

P<sub>3</sub> = Material sedimented at 50,000 x g.

P<sub>4</sub> = Material sedimented at 105,000 x g. P<sub>5</sub> = P<sub>2</sub> + P<sub>3</sub>.





between subcellular components of experimental and control strips were greater than those observed by Takagi, Akao and Takahashi (1965).

Takagi, Akao and Takahashi (1965), on the basis of their finding of the large difference in radioactivity in the mitochondrial and microsomal fractions, combined these fractions and used them in their subsequent fractionation studies, ignoring the radioactivity associated with other fractions.

A careful examination of all the data leads to the conclusion that it is unreasonable to proceed on this basis and ignore the radioactivity associated with the nuclear and the supernatant (S) fraction. This is particularly apparent in our data where approximately 50% of the total radioactivity is associated with the nuclear fraction. One interpretation of the above data is that the subcellular fractions are impure and a dibenamine-receptor complex, normally present in a particular location in the cell, is contaminating the other fractions. A second interpretation of the above data is that dibenamine hydrochloride in addition to labeling the  $\alpha$ -adrenergic receptors, located in a particular cellular component, is also labeling sites in other subcellular components. Since we could not decide from the above data in which subcellular component the  $\alpha$ -adrenergic receptor was located, this approach to the problem was not pursued further. It was decided that direct chemical fractionation of the labeled strips would be likely to be a more profitable approach to the problem of isolating the dibenamine-receptor complex. It is of interest that such studies have been utilized successfully by workers in the cancer field to isolate complexes formed between alkylating agents and tissue components (Brookes & Lawley, 1965; Connors et al., 1965).



CHAPTER V   LABELING STUDIES ON MODIFIED  
RABBIT AORTIC STRIPS



## Introduction

Since epinephrine in addition to protecting  $\alpha$ -adrenergic receptors also protects the uptake sites in sympathetic nerve terminals from combination with  $^{14}\text{C}$ -dibenzamine hydrochloride, a method was sought to remove these uptake sites. Recent studies by several groups of workers have suggested a method by which this may be accomplished. Histochemical studies of blood vessels revealed that the terminal sympathetic effector plexus is confined to the adventitio-medial junction (De La Lande, Frewin & Waterson, 1967; Fig. 32). Moreover, removal of the adventitia from rabbit aortic strips removes the bulk of the sympathetic nerve terminals and concomitantly the capacity of the tissue to bind  $^3\text{H}$ -norepinephrine (Eckhardt, Wastila & Maxwell, 1967). Bevan & Verity (1967) have described a procedure for the preparation of nerve-free rabbit aortic strips by selective mechanical destruction of the adventitia and adventitio-medial junction.

We have repeated procedure II with rabbit aortic strips from which the adventitia had been removed using either epinephrine or phentolamine as a protecting agent.

## Experimental

### i. Effect of Removal of Adventitia on the Response of Strips to Epinephrine, Norepinephrine, Tyramine and Cocaine

The response of rabbit aortic strips to epinephrine bitartrate ( $5 \times 10^{-8}$ ), norepinephrine bitartrate ( $5 \times 10^{-8}$ ) and tyramine hydrochloride ( $1 \times 10^{-5}$ ) was recorded. Cocaine hydrochloride ( $3 \times 10^{-6}$ ) was added to the baths and after 5 min the response of the strips to epinephrine bitartrate ( $5 \times 10^{-8}$ ) and norepinephrine bitartrate ( $5 \times 10^{-8}$ ) was recorded.



A

B

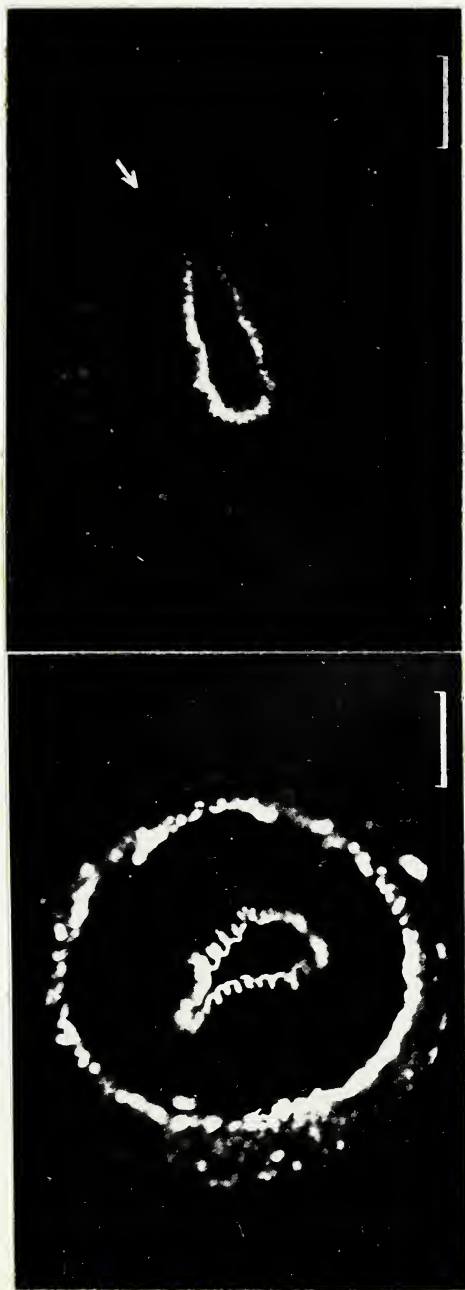


Fig. 32. The Appearance of a Section of an Innervated Artery (A), and of a Denervated Artery (B) from the Opposite Ear of the Same Rabbit (De La Lande, Frewin & Waterson, 1967). Both were treated simultaneously by the fluorescence histochemical method. In the denervated artery specific fluorescence is not present in the adventitio-medial border, the position of which is shown at one point by a white arrow. Scale: 100 $\mu$





The strips were placed with their adventitial surface facing upward on a piece of paper towel moistened with Krebs's bicarbonate solution. The adventitial layer of the strips was peeled off the media with great care by means of a fine curved forceps. During this procedure the strips were flooded continuously with Krebs's bicarbonate solution. The modified strips were mounted in organ baths and the responses to epinephrine, norepinephrine, tyramine and cocaine were recorded. Experiments of this type were repeated at least four times with similar results. Typical recordings are shown in Figs. 33 & 34. In order to shorten subsequent descriptions of this procedure, aortic strips from which the adventitia has been removed will be referred to as modified aortic strips.

#### ii. Histology

We are indebted to Mr. L. T. Chen of the Department of Zoology of the University of Alberta for preparing aortic sections for histological examination by the procedure described below: segments of modified rabbit aortic strips were fixed in Zenker-formal solution. Paraffin sections (6 $\mu$ ) were cut perpendicular to the tissue strips and stained with Mallory-Heidenhain staining solution (Cason, 1950). Segments of unmodified rabbit aortic strips were treated in the same manner. Photographs of the sections are shown in Fig. 35. Full details of the procedure are described in Appendix II.

#### iii. Labeling Studies with Modified Aortic Strips

##### (a) Epinephrine as Protecting Agent

Modified aortic strips were mounted in organ baths as shown in



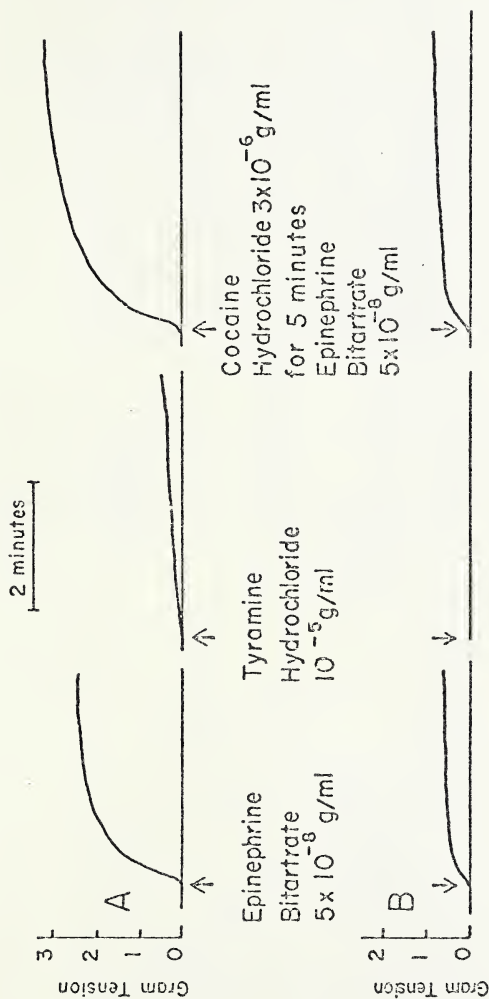


Fig. 33. The Effect of Removal of Adventitia on the Response of Rabbit Aortic Strips to Epinephrine, Tyramine, and Cocaine.  
A. Unmodified strip. B. Modified strip.



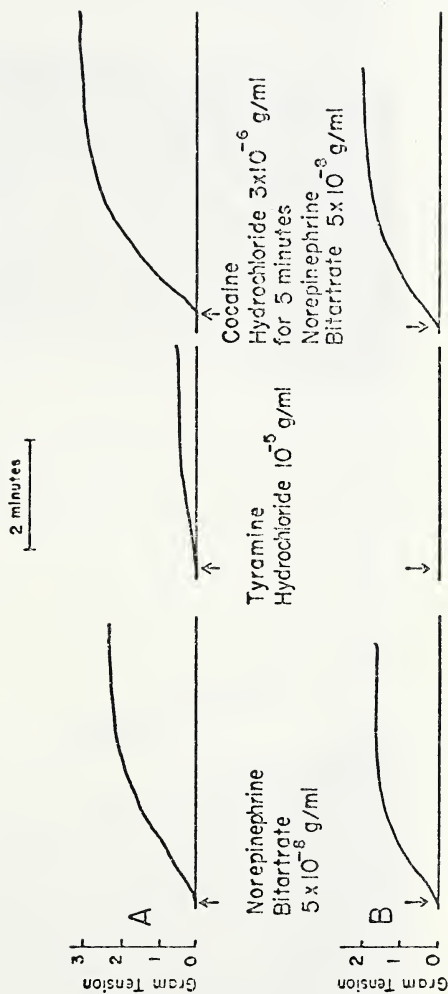
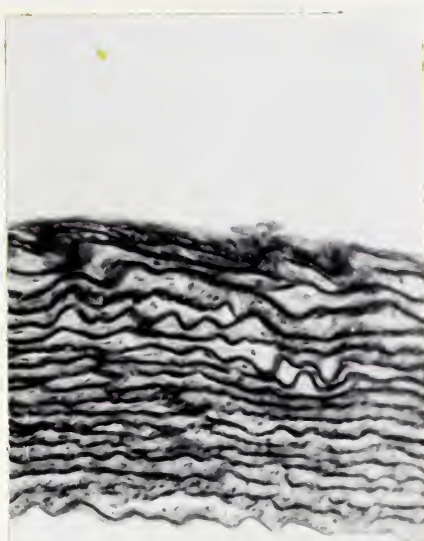


Fig. 34. The Effect of Removal of Adventitia on the Response of Rabbit Aortic Strips to Norepinephrine, Tyramine, and Cocaine. A. Unmodified strip. B. Modified strip.



B



A

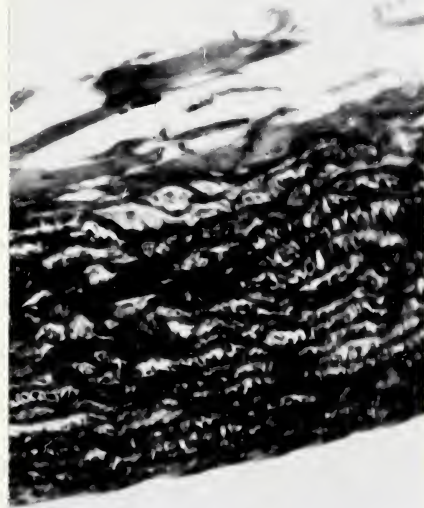


Fig. 35. Removal of Adventitia from Rabbit Aortic Strips. A. Section from unmodified strip. B. Section from modified strip. Mallory-Heidenhain, X430.





Fig. 9B, and the responses of the strips to several concentrations of epinephrine were recorded (Fig. 36A). The strips were exposed to epinephrine bitartrate ( $1 \times 10^{-4}$ ) for 5 min and  $^{14}\text{C}$ -dibenamine hydrochloride ( $3 \times 10^{-6}$ ) was added (Fig. 36B). After a further 20 min period, the strips were washed for 3 hr at 15 min intervals and the responses of strips to epinephrine redetermined (Fig. 36C). The radioactivity associated with lipid-extracts and lipid-free residues of the strips was determined. As controls the above experiments were repeated with the omission of epinephrine bitartrate ( $1 \times 10^{-4}$ ).

(b) Phentolamine as a Protecting Agent

The procedure described in (a) was repeated by substituting phentolamine methanesulfonate ( $1 \times 10^{-5}$ ) for epinephrine bitartrate ( $1 \times 10^{-4}$ ) as an  $\alpha$ -adrenergic receptor protecting agent (Fig. 37A,B,C).

Results and Discussion

Bevan & Verity (1967) used the following procedure for the preparation of nerve-free rabbit aortic strips: aortic strips were placed in flat-bottomed grooves cut in a plastic block and the adventitia projecting above the surface of the block was destroyed by means of a vibrating razor blade. Photographs of stained sections cut from strips prepared in the above manner are shown in Fig. 38. The bulk of the adventitia is shown to have been removed by this procedure. We have explored the possibility of obtaining nerve-free rabbit aortic strips by carefully peeling off the adventitia with a fine curved forceps. Photographs of stained sections obtained by this procedure are shown in Fig. 35. Our results appear to be



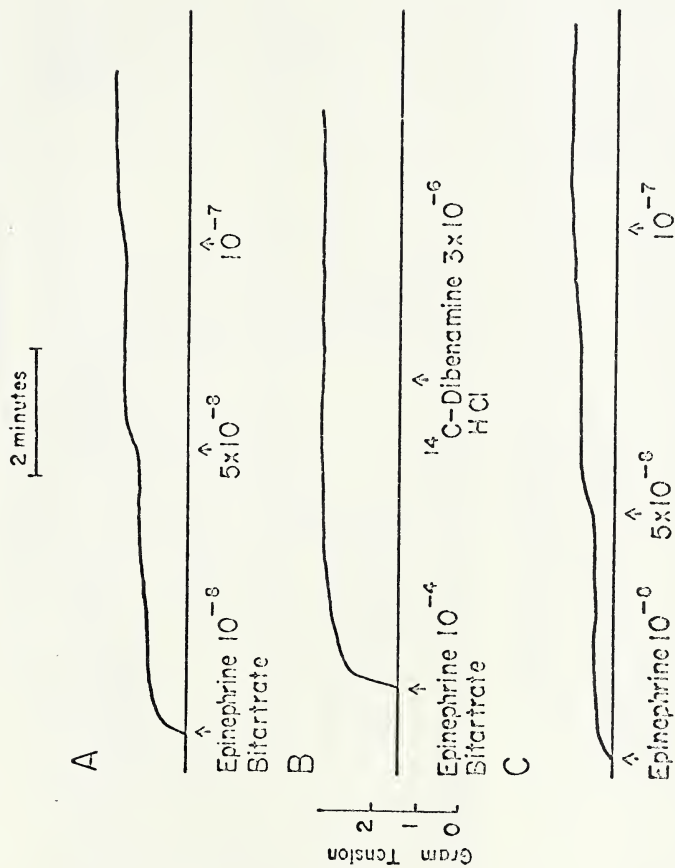


Fig. 36. The Response of Modified Rabbit Aortic Strips to Epinephrine in Labeling Studies Using Epinephrine as Protecting Agent.



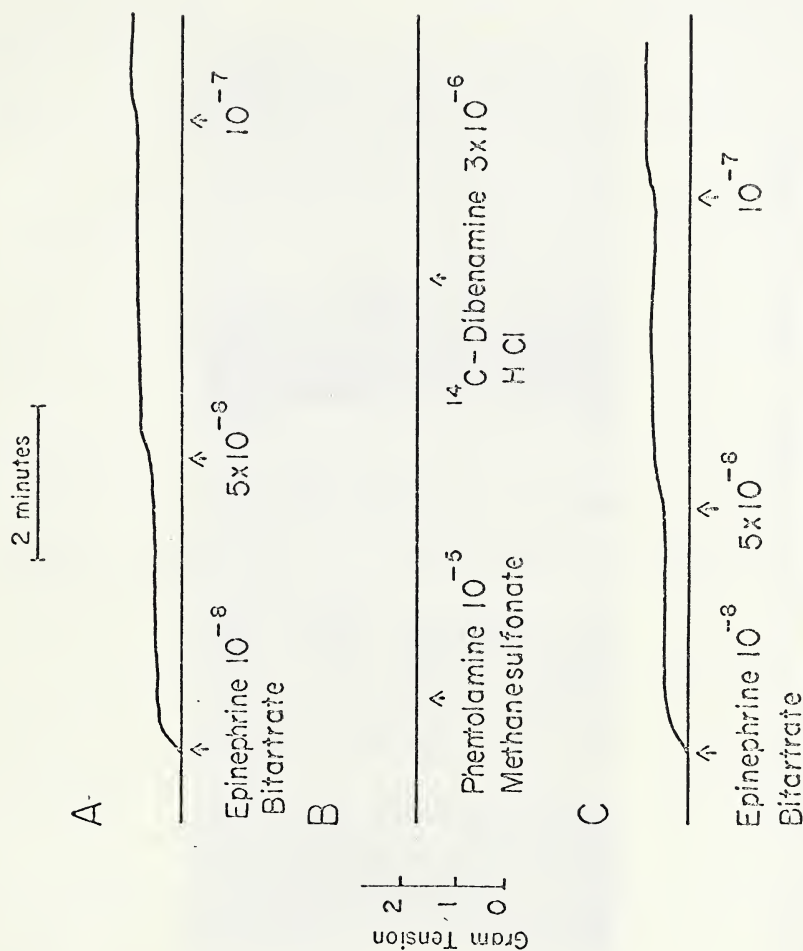


Fig. 37. The Response of Modified Rabbit Aortic Strips to Epinephrine in Labeling Studies Using Phentolamine as Protecting Agent.



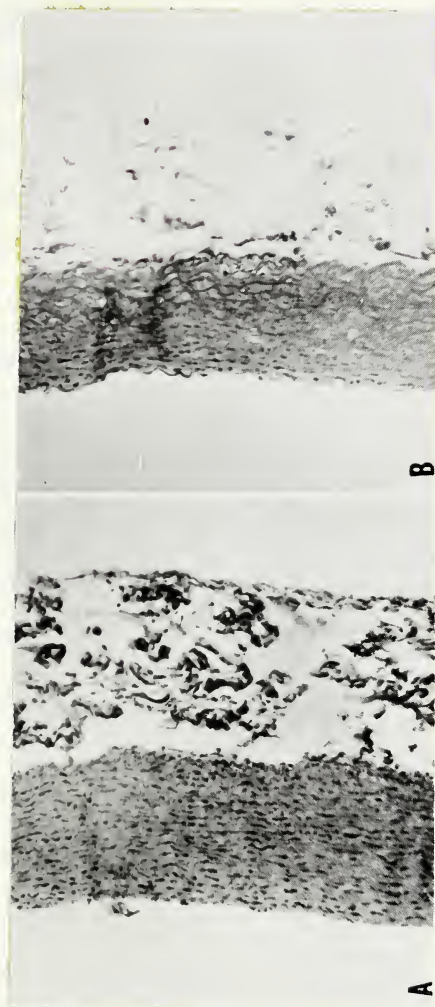


Fig. 38. Destruction of Adventitia and Adventitio-Medial Junction of Rabbit Aortic Strip after Denervation Procedure (Bevan & Verity, 1967).  
A. Control section after incubation in Krebs's solution showing approximate thickness of adventitia. B. After denervation. Masson trichrome, X180.





at least as good using this simple method as those obtained by Bevan and Verity.

It was of interest to compare the responses to a series of drugs in our modified aortic strips to those observed by Bevan and Verity in their nerve-free aortic strips. In both preparations the contractions elicited by tyramine in the unmodified aortic strips were not observed in the modified strips. Since tyramine produces contractions by liberation of norepinephrine from sympathetic nerve terminals in the adventitia it is clear that in these modified preparations the sympathetic nerve terminals are almost completely absent. Our modified strips responded to norepinephrine ( $5 \times 10^{-8}$ ) with a contraction which was 45% of that observed in the unmodified strips. In similar experiments Bevan & Verity found the response to a maximum dose of norepinephrine in modified strips to be 65% of that observed in unmodified strips.

Modified and unmodified rabbit aortic strips were treated with cocaine hydrochloride ( $3 \times 10^{-6}$ ). After 5 min the response of the strips to epinephrine and norepinephrine was recorded. The results shown in Table XV and Figs. 33 & 34 show that pretreatment of modified and unmodified strips with cocaine increases the sensitivity of the strips to norepinephrine. According to current interpretations of the action of cocaine this agent increases the sensitivity of smooth muscle to norepinephrine by preventing its uptake into sympathetic nerve terminals. For this reason the results obtained with norepinephrine, using unmodified strips pretreated with cocaine, were anticipated. However, the increased sensitivity of modified strips to norepinephrine after cocaine pretreatment is not in accord with the current interpretation of cocaine action, but accords with



TABLE XV. THE EFFECT OF COCAINE ON THE RESPONSE OF MODIFIED AND UNMODIFIED RABBIT AORTIC STRIPS TO NOREPINEPHRINE.

Drug (g/ml)	Contraction (g tension)			
	Unmodified Strip		Modified Strip	
	No Cocaine	Cocaine ( $3 \times 10^{-6}$ )	No Cocaine	Cocaine ( $3 \times 10^{-6}$ )
Norepinephrine Bitartrate $5 \times 10^{-8}$	$2.4 \pm 0.6$ (4)*	$3.3 \pm 0.5$ (4)	$1.1 \pm 0.6$ (4)	$1.3 \pm 0.8$ (4)
P Value	< 0.05		< 0.05	

\* Numbers enclosed by parentheses indicate the number of experiments performed.



results obtained by Bevan & Verity (1967) with nerve-free aortic strips. The latter workers concluded that the increased sensitivity of aortic strips to norepinephrine after cocaine is due to a presynaptic action and also to a direct action on vascular smooth muscle cells.

The results of the labeling studies with modified rabbit aortic strips using epinephrine ( $1 \times 10^{-4}$ ) as the protecting agent are recorded in Table XVI. A significant increment in radioactivity ( $P < 0.05$ ) was noted in the lipid-free residues of control strips when compared to experimental strips. However, no significant difference in radioactivity was noted on comparing the lipid extracts of control and experimental strips. With unmodified strips a significant increment in radioactivity was noted in both lipid extracts and lipid-free residues of control as compared to experimental strips (Chapter IIID). In comparing the results of these two experiments we conclude that the significant increment in radioactivity associated with the lipid-free residue probably represents the  $^{14}\text{C}$ -dibenzamine hydrochloride molecules combined with  $\alpha$ -adrenergic receptor sites. On the other hand the significant increment in the radioactivity associated with lipid extracts in unmodified aortic strips probably represents the  $^{14}\text{C}$ -dibenzamine hydrochloride molecules combined with uptake sites in nerve terminals.

The results of the labeling studies with modified aortic strips using phentolamine methanesulfonate ( $1 \times 10^{-5}$ ) as the protecting agent are recorded in Table XVII. No significant increment in radioactivity ( $P > 0.05$ ) was noted in either the lipid extracts or the lipid-free residues of control strips as compared to experimental strips. With unmodified aortic strips significant increments in radioactivity were noted in both lipid extracts



TABLE XVI. THE DISINTEGRATIONS PER MINUTE DETECTED IN THE LIPID EXTRACTS AND LIPID-FREE RESIDUES OF CONTROL AND EXPERIMENTAL MODIFIED RABBIT AORTIC STRIPS USING EPINEPHRINE AS A PROTECTING AGENT.

Remaining Sensitivity to Epinephrine (% of original)	Dry Weight of Aortic Strip (mg)		Dis/min in Lipid-Free Residue/mg Dry Weight of Strip		Dis/min in Lipid Extract/mg Dry Weight of Strip		Total dis/min/mg of Dry Weight of Strip	
	Control Strip	Experimental Strip	Control Strip	Experimental Strip	Control Strip	Experimental Strip	Control Strip	Experimental Strip
47	5.4	5.1	428	235	122	55	550	290
39	11.4	7.3	412	254	102	58	514	312
82	7.7	6.8	431	460	74	98	505	558
61	6.8	10.5	566	384	111	80	677	664
50	4.4	5.6	529	470	118	158	647	628
48	5.2	6.5	413	431	95	82	508	513
60	6.3	4.4	447	421	94	109	541	530
54	9.6	5.6	365	515	68	106	433	621
66	4.7	7.3	418	369	94	86	512	455
57	3.1	7.3	503	435	99	91	602	526
52	7.3	7.4	512	433	101	75	613	508
62	7.3	7.4	457	409	101	92	558	501
62	7.4	7.2	471	359	90	57	561	416
45	4.3	7.0	478	428	86	91	564	579
50	4.7	8.4	535	399	110	86	645	485
Mean $\pm$ S.D.	6.4 $\pm$ 2.1	6.9 $\pm$ 1.4	464 $\pm$ 54	400 $\pm$ 72	98 $\pm$ 14	88 $\pm$ 25	562 $\pm$ 63	488 $\pm$ 91
P Value	< 0.05		> 0.05		< 0.05		< 0.05	

The total dis/min/mg dry weight of strip is the sum of the dis/min/mg of lipid extract and lipid-free residue. 117







TABLE XVII. THE DISINTEGRATIONS PER MINUTE DETECTED IN THE LIPID EXTRACTS AND LIPID-FREE RESIDUES OF CONTROL AND EXPERIMENTAL MODIFIED RABBIT AORTIC STRIPS USING PHENTOLAMINE AS A PROTECTING AGENT.

Dry Weight of Aortic Strip (mg)		Dis/min in Lipid-Free Residue/mg Dry Weight of Strip		Dis/min in Lipid Extract/mg Dry Weight of Strip		Total dis/min/mg of Dry Weight of Strip	
Control Strip	Experimental Strip	Control Strip	Experimental Strip	Control Strip	Experimental Strip	Control Strip	Experimental Strip
3.8	7.7	221	476	53	92	274	568
5.9	8.6	505	282	94	57	689	339
9.8	11.3	388	409	90	85	482	494
13.4	7.4	300	399	72	80	372	479
7.0	5.2	362	352	83	74	445	426
3.2	7.1	417	369	107	75	524	444
5.4	7.3	594	478	128	103	722	581
8.1	5.0	490	629	97	142	587	771
3.4	5.5	303	361	66	61	369	422
3.7	2.6	243	570	68	94	301	664
4.3	3.9	320	479	80	93	400	572
3.4	5.3	498	443	134	96	632	529
4.6	5.2	498	420	120	83	618	503
5.0	5.7	416	389	97	76	513	465
Mean $\pm$	6.3 $\pm$	361 $\pm$	433 $\pm$	92 $\pm$	87 $\pm$	495 $\pm$	518 $\pm$
S.D. 2.8	2.1	144	87	23	20	137	105
P Value	> 0.05		> 0.05		> 0.05		> 0.05

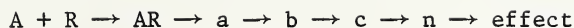
The total dis/min/mg dry weight of strip is the sum of the dis/min/mg of the lipid extract and lipid-free residue.



and lipid-free residues of control strips when compared to experimental strips (Chapter III E). This finding was unexpected since phentolamine had conferred almost 100% protection against blockade by  $^{14}\text{C}$ -dibenamine hydrochloride in both modified and unmodified strips. In comparing the results obtained with epinephrine and phentolamine as protecting agents it is of interest to consider the molar ratios of these substances employed relative to  $^{14}\text{C}$ -dibenamine hydrochloride. Thus the molar ratio of epinephrine to  $^{14}\text{C}$ -dibenamine hydrochloride was 33:1, while the molar ratio of phentolamine to  $^{14}\text{C}$ -dibenamine hydrochloride was 2.6:1. Bearing this point in mind there are three possible explanations for the unexpected results obtained with phentolamine, viz., (1) phentolamine ( $1 \times 10^{-5}$ ) protects only a fraction of the total receptors from combination with  $^{14}\text{C}$ -dibenamine hydrochloride and therefore no significant difference in radioactivity between control and experimental strips is observed. However, the small number of receptors protected from combination with  $^{14}\text{C}$ -dibenamine hydrochloride suffices to give a maximum response when occupied by epinephrine; (2) phentolamine ( $1 \times 10^{-5}$ ) protects the total receptor population from combination with  $^{14}\text{C}$ -dibenamine hydrochloride. However, the total number of receptors is so small that no significant difference in radioactivity is detectable between control and experimental modified strips by our technique. The significant difference in radioactivity between control and experimental modified strips observed with epinephrine would then be explained by the fact that epinephrine protects large numbers of non-specific sites as well as  $\alpha$ -adrenergic receptor sites from combination with  $^{14}\text{C}$ -dibenamine hydrochloride; (3) Moran (1967) has pointed out that except for the glycogenolytic response to adrenergic stimuli we know little of the steps between



receptors and final effects:



where A = agonist, R = receptor, a,b,c,n = reaction steps. Moran (1967) cites evidence to show that two antagonists might block an adrenergic effect by action at two different sites in the sequence or by one drug acting at a site in the sequence and the other acting outside of the sequence influencing the adrenergic effect indirectly. A possible explanation for our results can be provided if it is assumed that the two antagonists, phentolamine and dibenamine hydrochloride act at different sites. Since there is good evidence that dibenamine hydrochloride acts at the same site as epinephrine (Belleau, 1958) it must be assumed that phentolamine acts at a different site. Thus it is possible that phentolamine, acting outside of the sequence, protects the  $\alpha$ -adrenergic receptor from inactivation by dibenamine hydrochloride by inducing a conformational change in the receptor molecule. However, in inducing this conformational change it might expose new sites for combination with  $^{14}\text{C}$ -dibenamine hydrochloride and thus no significant difference in radioactivity is observed between control and experimental modified strips.



## CHAPTER VI    GENERAL DISCUSSION





Recently three important papers have appeared in which labeling studies similar to those reported in this thesis have been described (Lewis & Miller, 1966; May et al., 1967; Moran et al., 1967). It is the purpose of this discussion to summarize our results and compare the results with those reported by these workers.

Moran et al. (1967) have attempted to specifically label the  $\alpha$ -adrenergic receptors of rabbit vas deferens and aorta, and a variety of amines were used to protect the strips from combination with the irreversible  $\alpha$ -adrenergic blocking agent  $^3\text{H-N-(2-bromoethyl)-N-ethyl-N-1-naphthyl-methyl-amine}$  ( $^3\text{H-SY.28}$ ; Fig. 5). In addition a new procedure was developed using a short lasting irreversible  $\alpha$ -adrenergic receptor antagonist, viz., N,N-dimethyl-2-bromo-2-phenylethylamine (Fig. 39) to protect the receptor against

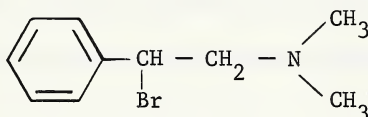


Fig. 39. The Chemical Structure of N,N-Dimethyl-2-bromo-2-phenylethylamine

irreversible blockade by  $^3\text{H-SY.28}$ . They obtained the following results: (1) the uptake of  $^3\text{H-SY.28}$  by rabbit vas deferens and aorta was non-specific and this agent showed little specificity for  $\alpha$ -adrenergic receptors; (2) pretreatment of rabbit aorta and vas deferens with N,N-dimethyl-2-bromo-2-phenylethylamine did not afford any significant protection against the uptake of  $^3\text{H-SY.28}$ ; (3) several sympathomimetic amines afforded protection against the uptake of  $^3\text{H-SY.28}$  in these tissues. However, no correlation was found between the protective ability of these compounds and their direct or indirect



sympathomimetic activities. These workers conclude, "It appears that while it is possible to obtain receptor protection and specificity of action with irreversibly acting antagonists at a pharmacologic level, it is not possible to obtain specificity of action at the chemical level with the agents currently available." A careful analysis of the data of these workers indicates that insufficient experiments were carried out with individual protecting agents in order to draw final conclusions. It is worth pointing out that had we restricted the number of experiments to the number carried out by Moran et al. (1967) we too would often not have observed significant differences where they in fact existed. These workers stressed the fact that the protective action of norepinephrine and many other amines will be exerted at both the receptor and amine uptake sites so that ambiguity of interpretation arises. In our studies with modified aortic strips we have removed the amine uptake sites thus avoiding ambiguity in the interpretation of our work.

Our results lead to the conclusion that the  $\alpha$ -adrenergic receptor site is not lipid in nature as claimed by Dikstein & Sulman (1965). Lewis & Miller (1966) used a procedure, analogous to our procedure II, to label the  $\alpha$ -adrenergic receptors of rat seminal vesicle with  $^3\text{H}$ -phenoxybenzamine hydrochloride. These workers came to the following conclusions: (1) either norepinephrine ( $1 \times 10^{-4}$ ) or phentolamine ( $1 \times 10^{-6}$ ) can be used to protect the  $\alpha$ -adrenergic receptors of rat seminal vesicle from combination with  $^3\text{H}$ -phenoxybenzamine; (2) the radioactivity associated with the lipid extract of labeled tissues was approximately 37% of that present in whole tissues; (3) a significant increment in radioactivity was found in the lipid-free residue of the control vesicle when compared to the experimental



vesicle. However, no significant difference was noted in the lipid extract of the control and experimental vesicle; (4) the lipid-free residue is probably concerned with  $\alpha$ -adrenergic receptor interaction; lipids are not involved in  $\alpha$ -adrenergic receptor interaction. These observations are in essential agreement with those which we have obtained.

Lewis & Miller (1966) have pointed out that the difference in lipid-free residue radioactivity between phentolamine-protected and non-protected preparations should give an upper estimate of the number of  $\alpha$ -adrenergic receptors. On this basis these workers have estimated that there are  $1.7 \times 10^{13}$   $\alpha$ -adrenergic receptors per gram of dried rat seminal vesicle. It was of interest to compare the number of  $\alpha$ -adrenergic receptors in rabbit aorta to the number in rat seminal vesicle. This was done by using the difference in radioactivity between lipid-free residues of modified control and experimental strips (Table XVI) to estimate the total number of  $\alpha$ -adrenergic receptors in rabbit aorta as follows:

- |  |  |
|--|--|
| (1) The difference between the radioactivity associated with the lipid-free residues of modified control and experimental strips   | = (464-400) dis/min/mg dry weight of strip                     |
|  | = 64 dis/min/mg dry weight of strip                            |
| (2) Specific activity of $^{14}\text{C}$ -dibenamine hydrochloride   | = $3567 \times 10^6$ dis/min/g                                 |
| (3) Molecular weight of $^{14}\text{C}$ -dibenamine hydrochloride  | = 296  |
| (4) The difference between the number of gram molecules of $^{14}\text{C}$ -dibenamine hydrochloride associated with the lipid-free residues of modified control and experimental strips | = $64 / (3567 \times 10^6 \times 296)$                         |
|  | = $6.05 \times 10^{-11}$ gram molecules/mg dry weight of strip |





- (5) Avogadro's number  $= 6.02 \times 10^{23}$
- (6) The difference between the number of molecules of  $^{14}\text{C}$ -dibenamine hydrochloride associated with the lipid-free residues of modified control and experimental strips  $= 6.05 \times 10^{-11} \times 6.02 \times 10^{23} \times 10^3$  molecules/g dry weight of strip

Assuming that one molecule of  $^{14}\text{C}$ -dibenamine hydrochloride combines with one receptor, the number of  $\alpha$ -adrenergic receptors is estimated to be  $3.6 \times 10^{16}$  per gram dry weight of aorta. From the data in Table XII and Fig. 29 (Chapter IIIF) we observe that the minimum amount of  $^{14}\text{C}$ -dibenamine hydrochloride required to completely block all the  $\alpha$ -adrenergic receptors in rabbit aortic strips is  $6.3 \times 10^{-11}$  gram molecules per milligram dry weight of strip. This information can be used to calculate the number of receptors per gram dry weight of aorta. The figure of  $3.8 \times 10^{16}$  receptors obtained is in close agreement with that derived from the protection experiment discussed above. This result supports the view of Furchgott (1966) that despite criticism of protection experiments the technique is useful in differentiating receptors.

The number of  $\alpha$ -adrenergic receptors in rabbit aorta is approximately 2,000 times higher than that estimated by Lewis & Miller (1966) for the number in rat seminal vesicle. May et al. (1967) estimated the number of  $\alpha$ -adrenergic receptors by determining the amount and time course of the loss of radioactivity from rabbit aortic strips treated with  $^3\text{H}$ -N,N-dimethyl-2-bromo-2-phenylethylamine and correlating this loss of radioactivity with the response of the strips to norepinephrine. Their estimate of  $1.15 \times 10^{15}$  receptors per gram of tissue dry weight is in fair agreement with the figure we have obtained.





It is of interest to compare the figure we have obtained for the number of  $\alpha$ -adrenergic receptors in rabbit aorta with those obtained by other workers for a variety of tissue receptors (Table XVIII).

Investigators in the cancer field studying the mechanism of action of alkylating agents are beset by almost identical difficulties to those we have encountered in our studies. This is illustrated by the following statement from Connors et al. (1965):

Available evidence indicates that the alkylating agents almost certainly cause tumor inhibition as a result of alkylation of intracellular sites, and there appears to be no doubt that even at physiological dose levels many different sites are alkylated. The problem is to decide which alkylation leads to tumor inhibition.

These workers have introduced a very interesting new approach to the solution of this problem. They have compared the cellular sites which two tritium labeled mustards alkylate in relation to the degree of tumor inhibition produced. Their rationale for the study was as follows:

It was believed that if alkylation of any site could be correlated with tumor inhibition on a dose-response basis, then it would be possible to conclude that alkylation of such a site was necessary for tumor inhibition.

By this approach they were able to show that tumor inhibition was unrelated to either the total uptake of drug by the tumor or the total amount bound to protein. On the other hand for both drugs comparable tumor inhibition occurred when there was a similar level of radioactivity associated with either the deoxyribonucleic acids (DNA) or the ribonucleic acids (RNA). We believe that this approach, using  $^{14}\text{C}$ -dibenamine and  $^{14}\text{C}$ -phenoxybenzamine could be adapted to determine which alkylation sites in rabbit aortic strips lead to  $\alpha$ -adrenergic receptor blockade.



TABLE XVIII. THE ESTIMATED NUMBER OF  $\alpha$ -ADRENERGIC AND CHOLINERGIC RECEPTORS FOR VARIOUS TISSUES.

Type of Receptor	Tissue	Number of Receptor $\times 10^{14}/g$ Dry Weight	Method for Estimation	Reference
$\alpha$ -Adrenergic Receptor	Rat Seminal Vesicle	0.17	Labeling studies based on receptor protection using $^3H$ -phenoxybenzamine	Lewis & Miller (1966)
	Rabbit Aorta	11.5	Measuring the loss of radioactivity from tissue treated with $^3H$ -N,N-dimethyl-2-bromo-2-phenylethylamine	May et al. (1967)
	Rabbit Aorta	360	Labeling studies based on receptor protection using $^{14}C$ -dibenzamine	This thesis
Cholinergic Receptor	Electric Organ of Electric Eel	34	Calculation based on data of Waser (1961) and other workers	Trams (1964)
	Guinea-Pig Intestinal Smooth Muscle	5	Measuring the kinetics of $^3H$ -atropine uptake	Paton & Rang (1965)
	Frog's Heart	1	50% inhibition of contractile response	Clark (1933)



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APPENDIX I. Statistical Analysis

The t-value for the unpaired t-test was calculated according to the following equation (Ferguson, 1959):

$$t = \bar{X}_1 - \bar{X}_2 / \sqrt{S^2/N_1 + S^2/N_2}$$

where  $\bar{X}_1$  = the mean of the experimental samples

$\bar{X}_2$  = the mean of the control samples

$N_1$  = the number of experimental samples

$N_2$  = the number of control samples

$S^2$  = the unbiased estimate of the population variance

$$= (X - \bar{X}_1)^2 + (X - \bar{X}_2)^2 / (N_1 + N_2 - 2)$$

where  $(X - \bar{X}_1)^2$  = the sum of squares of deviations about the mean of experimental samples

$(X - \bar{X}_2)^2$  = the sum of squares of deviations about the mean of control samples

The probability of 0.05 was selected as the point of significance. Since in our experimental design (procedure I) it was expected that experimental strips would have greater radioactivity than control strips, a one-tailed test of significance was used. Similarly with procedure II it was expected that control strips would have greater radioactivity than experimental strips and a one-tailed test of significance was used.

For test of significance other than that described above, the paired t-test was used (Ferguson, 1959). The probability of 0.05 was selected as the point of significance and two-tailed test of significance was used.



APPENDIX II. Variation of Time of Exposure of Aortic Strips to  
<sup>14</sup>C-Dibenamine Hydrochloride (Procedure II)

It was of interest to determine whether exposure of strips to <sup>14</sup>C-dibenamine hydrochloride for 20 min was the most suitable period in labeling procedure II. The results in Fig. 40 indicate that there was no advantage in either increasing or decreasing the period of exposure to this agent.



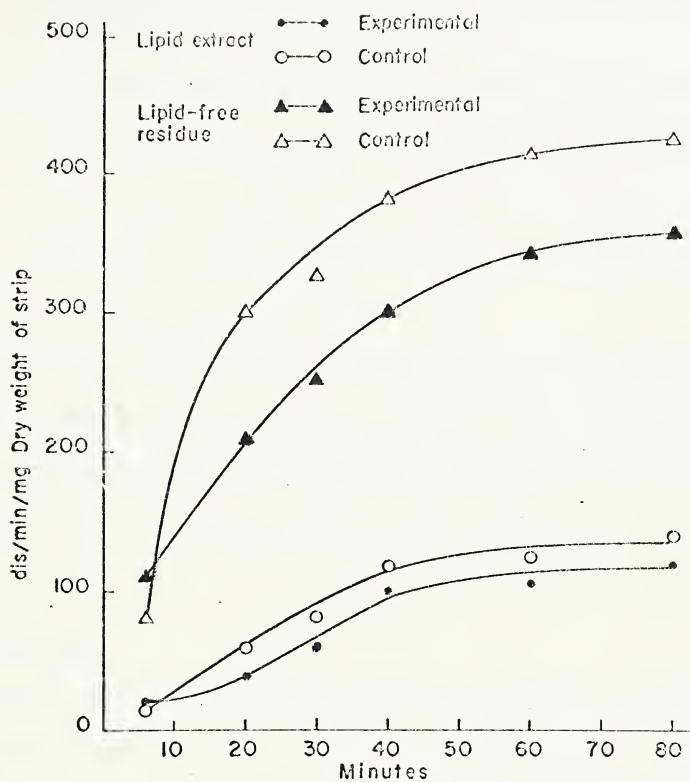


Fig. 40. Variation of Time of Exposure of Aortic Strips to  $^{14}\text{C}$ -Dibenamine Hydrochloride (Procedure II).



APPENDIX III. Histology of Rabbit Aortic Tissuei. Solutions

- (a) Bouin's fluid was prepared by mixing 75 ml of saturated aqueous picric acid, 25 ml of formalin and 5 ml of acetic acid.
- (b) Zenker-formal solution was prepared by dissolving 5 g of mercuric chloride, 2.5 g of potassium dichromate and 1 g of sodium sulfate in 100 ml of distilled water. Formalin in the quantity of 5 ml was added to the mixture immediately before use.
- (c) Mallory-Heidenhain staining solution was prepared by dissolving 1 g of phosphotungstic acid crystals, 2 g of orange G, 1 g of aniline blue and 3 g of acid fuchsin in 200 ml of distilled water.

ii. Procedure

Rabbit aortic strips were preserved in Bouin's fluid and then fixed in Zenker-formal solution. The strips were embedded in paraffin and a series of sections (thickness 6 $\mu$ ) was cut perpendicular to the tissue. After deparaffinization in xylene, the sections were passed through graded alcohol containing 0.25% iodine. The sections were then stained for 5 min in Mallory-Heidenhain solution and washed in running tap water for a few seconds. After completing the staining procedure the sections were rapidly dehydrated through graded alcohols and mounted in balsam.















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